The role of anaphylatoxins in asthma and airway remodelling

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Author's Declaration

I declare this thesis to have been composed entirely by myself, and the work contained herein to have been principally conducted by myself with the following exception:

Recruitment of subjects was performed by the respiratory research nurses in the Department of Asthma, Allergy and Respiratory Science at Guy's Hospital.

Abstract

C3a and C5a anaphylatoxins are proinflammatory polypeptides released during complement activation. They exert their biological functions by interacting with the G protein-coupled receptors, C3aR and C5aR respectively. Activation of the complement system has been implicated in the pathogenesis of many inflammatory diseases including asthma. Little is known, however, about the expression and location of complement components in asthmatic airways.

Experiments described in this thesis demonstrate the expression and localisation of certain complement components and their two receptors in the bronchial mucosa. C3a and C5a-stimulated production of remodelling mediators and the biological function of the anaphylatoxins on the structural cells was also assessed.

Immunohistochemical analysis revealed elevated expression/deposition of C3 and C5 components in the epithelium, airway smooth muscle and submucosa of asthmatics compared with controls, and also demonstrated expression of the two complement receptors on airways structural cells, including airway epithelial cells, endothelial cells, fibroblasts and smooth muscle cells (SMC).

In general C3a was the more effective of the two anaphylatoxins in inducing structural cellular proliferation: C3a increased fibroblast and endothelial cell proliferation at a range of concentrations embracing the physiological, although it increased SMC proliferation only at the highest concentration (10^{-7} M) employed, and epithelial cell proliferation only at the lowest concentration (10^{-9}

M) employed. C5a induced fibroblast proliferation but had no effect in this regard on the other structural cells.

The anaphylatoxins induced expression of various fibroblast growth factors (FGFs) and their receptors by the structural cells at the mRNA level: FGF2 was induced in all four cell types; FGFR1 and FGFRL1 were induced in all except SMC cells; FGFR4 was induced in SMC and endothelial cells; FGF11 was induced in fibroblast cells and FGF17 in SMC.

The structural cell types were observed to release FGF2 protein spontaneously. In contrast to their effects on early mRNA synthesis, however, the anaphylatoxins effected a net inhibition of FGF2 protein release by cultured epithelial cells, SMC and endothelial cells. With fibroblasts, C3a increased while C5a decreased FGF2 production.

The possible effects of C3a and C5a on the expression and release of IL-6 and IL-8 by airways structural cells was examined. IL-6 was released spontaneously only by airway fibroblasts and this release was unaffected by both anaphylatoxins. IL-8 was induced by C3a in fibroblasts and by C5a in endothelial cells.

The data support the hypothesis that complement components participate in acute or chronic asthmatic inflammatory processes, resulting in airway structural changes known as remodelling.

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Dedication

For my Mum & Dad.

You both saw this day from the beginning and believed I would get here,

This achievement is as much yours as mine

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List of Abbreviations

AHR	Airway hyperresponsiveness
ASM	Airway smooth muscle
BAL	Bronchoalveolar lavage
BSMC	Bronchial smooth muscle cell
C3aR	C3a receptor
C5aR	C5a receptor
cDNA	Complementary DNA
DAB	Diaminobenzidine
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid solution salt
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal trophic
FBS	Foetal bovine serum
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
HASM	Human airway smooth muscle cells
HBEpC	Human bronchial epithelial cells
HBSMC	Human bronchial smooth muscle cells
HBSS	Hanks balanced salt solution
HPFC	Human pulmonary fibroblast cells
HPMEC	Human pulmonary microvascular endothelial cells
HSMC	Human smooth muscle cell
HSPG	Heparan sulphate proteoglycans
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-1
ICC	Immunocytochemistry
Ig(E)	Immunoglobulin (E)
IHC	Immunohistochemistry
IL-(4)	Interleukin-(4)
MAC	Membrane attack complex
MAP	Mitogen-activated protein
MBL	Mannose-binding lectin
MCP-3	Monocyte chemoattractant protein
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
PAP	Peroxidase anti-peroxidase
PBS	Phosphate buffered saline
qPCR	Quantitative polymerase chain reaction
rbm	Reticular sub-basement membrane
RNA	Ribonucleic acid
STAT6	Signal transducer and activation of transcription-6
TARC	Thymus- and activation-regulated chemokine
TGF-β	Transforming growth factor beta
Th1	Type 1 helper T cell
T-TBS	Tween-tris buffered saline
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
α-SMA	αlpha-smooth muscle actin

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Chapter 1: Introduction

1.1 Pathogenesis of Asthma

Asthma is a complex airway inflammatory disease characterised by chronic inflammation, bronchoconstriction, airway hyperresponsiveness (AHR) and remodelling (Ali & Panettieri 2005).

It is one of the most common disorders encountered in clinical medicine in both adults and children (Renauld 2001). Many developed countries are in the midst of an epidemic of childhood asthma. According to the US 2002 National Health Interview Survey data, more than 8.9 million children younger than the age of 18 have had a diagnosis of asthma (Freidin & Timmermans 2008) and reported incidence is increasing dramatically in many developed nations (Renauld 2001).

There is a range of asthma severity, which tends to be maintained throughout life but may change for better or worse with age. Most patients with asthma are atopic (extrinsic asthma), but 10-20% are non-atopic (intrinsic asthma), and these patients often have a more severe form of the disease. Approximately 5% of patients have severe asthma that is difficult to control with maximal inhaler therapy and for whom new therapeutic approaches are needed (Barnes 2008).

As with all chronic inflammatory diseases, asthma is thought to arise from interaction between genetic background and the environment, involving allergens, viruses, bacteria and environmental and occupational pollution (Bai & Knight 2005). These factors contribute to inflammation, which results in both airway structural change and functional muscle change, leading to excessive airway narrowing followed by the symptoms of asthma (Bai & Knight 2005).

Despite asthma being multifactorial in origin, atopy, the genetic predisposition for the development of an immunoglobulin (Ig)E-mediated response to common aero-allergens, is the strongest identifiable predisposing factor for the development of asthma (Wills-Karp 1999). Although asthma is often accompanied by increased concentrations of circulating IgE, it has been difficult to demonstrate a precise role for IgE in the pathogenesis of asthma using murine models (Mehlhop *et al.* 1997). Nevertheless the benefits of anti-IgE strategies such as omalizumab argue empirically for a functional role for IgE at least in asthmatics who are atopic.

Airway obstruction in asthma is variable and reversible (Barnes 2008). There are structural changes in the airways of asthmatics, which include angiogenesis, epithelial fragility, enlarged submucosal mucus glands, goblet cell hyperplasia, increased matrix deposition in the airway wall, increased airway smooth muscle mass, wall thickening and abnormalities in elastin (Bai & Knight 2005). A point to note is that all components of the airway wall have been reported to be thickened in asthma (Renauld 2001). Contraction of the airway smooth muscle (ASM) is the principal component underlying the bronchoconstriction that characterises the acute phase of an asthmatic attack (Renauld 2001), but thickening of the airway wall will exaggerate the resulting airways obstruction.

It is thought that the observed structural alterations are generated as a consequence of chronic inflammation and contribute to the symptoms and physiological dysregulation seen in asthma (Renauld 2001). However, although symptoms may be well controlled, aspects of asthma pathology such as airway remodelling may not be prevented or reversed.

1.1.1 Chronic Inflammation

Chronic inflammation of the airways appears invariably to accompany the clinical syndrome of asthma. Inflammation seen in asthma is mainly located in the larger conducting airways, and although small airways can be affected in more severe forms of the disease, the lung parenchyma is not affected (Barnes 2008).

Asthmatic lungs typically show hyperinflation, mucus plugging in the airways, clusters of sloughed epithelial cells, and crystalline precipitates of eosinophil derived proteins (Renauld 2001).

In asthma there is chronic inflammation of the respiratory tract, which is mediated by the increased expression of multiple inflammatory proteins, including cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors. In acute episodes or exacerbations the intensity of this inflammation increases (Barnes 2008). Persistent inflammation in bronchial tissues, along with the ensuing activation of epithelial, subepithelial, and smooth muscle cells, presumably results in the structural changes known as airway remodelling (Pepe *et al.* 2005). Chronic uncontrolled airways inflammation and airway remodelling remain the suggested mechanisms by which severe persistent asthma develops (Pepe *et al.* 2005).

1.1.2 Airway Remodelling

The term remodelling describes the structural changes seen in the airways of patients with respiratory disease. The structural changes in the airway include angiogenesis (Busse *et al.* 1999), deposition of fibrous and other extracellular matrix (ECM) proteins, elevated smooth muscle mass and proliferation,

myofibroblast differentiation and proliferation, goblet cell hyperplasia and neovascularisation, subepithelial fibrosis, smooth muscle hypertrophy (Okayama, Ra, & Saito 2007) (Figure 1.1). Ongoing inflammation, airway injury, and healing are also part of the remodelling process in asthma (Cohen *et al.* 2007). The changes of remodelling also include thickening of the reticular sub-basement membrane (rbm), a significant feature of established asthmatic remodelling, increased numbers of submucosal glands and increased airway wall collagen (Bush 2008). Thickening of the epithelial rbm has been reported in both adults and school-aged children. However, it is not known at what age rbm thickening begins (Saglani *et al.* 2006). Nevertheless the extent of airway remodelling correlates with severity of asthma, at least in cross-sectional studies.

Airway remodelling is often considered to contribute to the element of irreversible airflow obstruction, which is a feature of some patients with asthma (Saglani *et al.* 2006). It has been suggested that reversibility in asthma is associated with mild disease but with disease progression, varying degrees of remodelling of the airways might lead to irreversibility (Pepe *et al.* 2005).

Overall, it can be seen that airway remodelling indicates changes in the composition, quantity as well as organisation of both the cellular and molecular components of the airway wall; alterations considered secondary to chronic injury and repair of the airway epithelial-mesenchymal trophic (EMT) unit (Evans *et al.* 1999). Nevertheless much of the literature on airway remodelling is speculative in the sense that any observed histological change are not proven to correlate with changes in physiology and do not correlate with clinical phenotype (Bai & Knight 2005).

Many mediators derived from various inflammatory cells (including mast cells, eosinophils, neutrophils and others) are implicated in the pathogenesis of airway remodelling, and consequently in the process of asthma. These mediators could be working in an autocrine or paracrine fashion. Although the mode of mechanism or signalling has not been investigated here, it is believed that the various mediators and components could be linked and work to either upregulate or downregulate/suppress one another or aid in recruiting infiltrating cells, which results in the features of airway remodelling. For instance, human mast cell mediators that modulate airway remodelling include tryptase, which has an effect on proliferation of fibroblasts; chymase, which degrades basement membrane components; and transforming growth factor beta (TGF-β) which affects proliferation of ASM cells (Okayama, Ra, & Saito 2007).

A number of studies have demonstrated a variety of structural changes in asthma that are likely not reversible, including subepithelial fibrosis, smooth muscle hypertrophy, goblet cell and blood vessel hyperplasia, which led to the use of the term 'remodelling.' Cohen et al have shown that in subjects with chronic asthma, there is a progressive decline in lung function that is greater than in normal subjects (Cohen *et al.* 2007).

As seen by most conducted studies and investigations, bronchoscopy and bronchial biopsy are the most commonly used techniques for determining the extent of airway remodelling (Lazaar & Panettieri 2003). However, there are still areas involving asthma and remodelling that are unclear, therefore understanding the pathogenesis and mechanisms driving airway remodelling could lead to new approaches to therapy and, specifically, retard the progressive decline in lung function that starts in asthma at an early stage (Cohen *et al.* 2007).

Inflammation and remodelling commonly co-exist but their natural histories are uncertain. It is uncertain how changes termed “remodelling” impact on lung physiology, although thickening of the mucosal layer and increased smooth muscle contractility could in theory contribute to symptoms of asthma. It is not certain whether remodelling changes are cumulative or sometimes reversible, although it could be hypothesised that there are structural changes such as fibrosis and ASM which are likely not reversible.

Airway inflammation precedes airway remodelling in animal models of asthma (Southam *et al.* 2007). Nonetheless, both clinical and animal studies indicate that the relationship between inflammation and remodelling is complex, and still incompletely understood (Dekkers *et al.* 2009). The presence of airway inflammation in patients with asthma is no guarantee at all for the occurrence of airway remodelling, and there is no clear correlation between the degree of inflammation and the degree of remodelling (Benayoun *et al.* 2003).

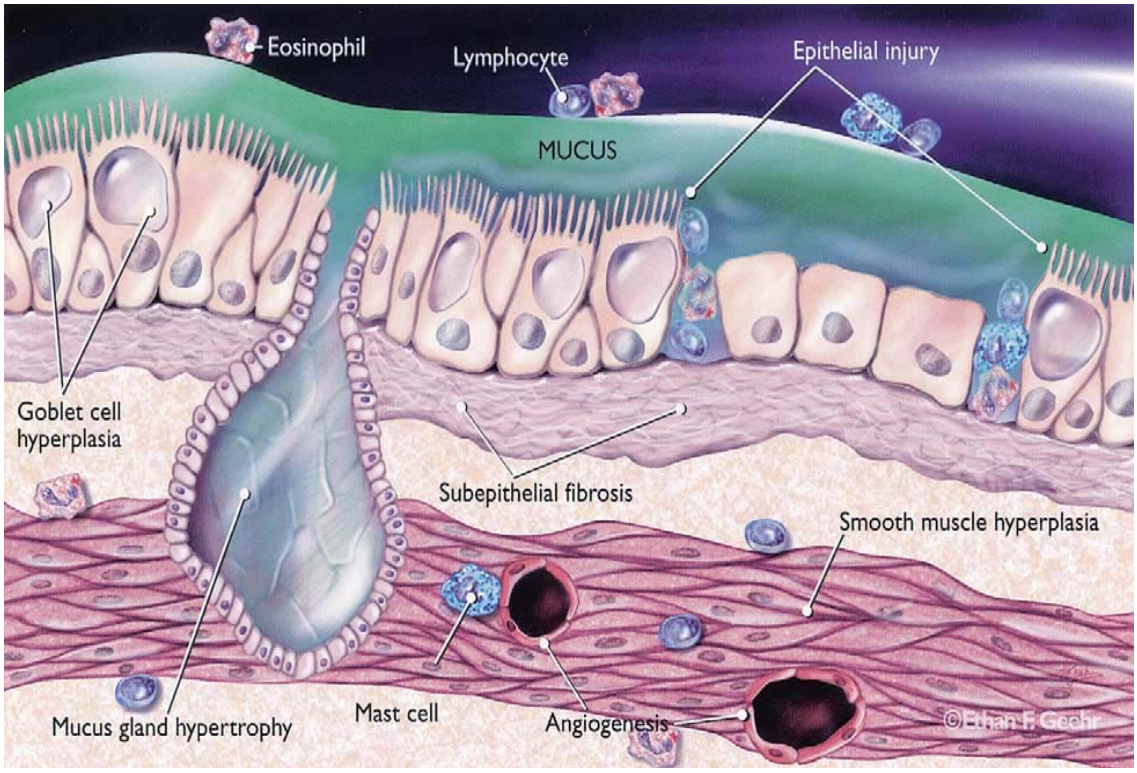

Figure 1.1 Components of airway remodelling (Lazaar & Panettieri 2003).

1.2 Complement System

In the late 19th century Jules Bordet discovered “complementing” antibodies in the plasma that recognised and eliminated pathogens. He described a heat-labile activity in serum that could complement the ability of a specific antibody to cause lysis of bacteria. Today this heat-labile activity is known to comprise dozens of serum proteins and is known as the complement system. The complement system contributes to the defence against pathogens (e.g bacteria, virus-infected cells and parasites) through release of anaphylatoxins, opsonisation and lysis of pathogens, stimulation of leukocyte chemotaxis and activation of leucocytes releasing inflammatory molecules.

Complement is known to function both in the defence against microbes and in the clean-up of tissues, e.g. in the disposal of immune complexes and apoptotic cells. To exert its biological activities, the complement system needs to be activated, after which it generates a variety of functional molecules. The complement system is composed of more than thirty plasma proteins, glycoproteins as well as soluble or membrane bound receptors (Guo & Ward 2005). A number of complement proteins are proteolytic enzymes that also in themselves become activated by proteolytic cleavage. This protein system acts as an enzymatic cascade via various protein-protein interactions. The pattern of sequential activation produces an expanding cascade of activity, which means that the activation of a single molecule will lead to thousands of molecules being generated in the following steps. Thus, many regulatory mechanisms are needed to prevent uncontrolled complement activation and tissue damage.

There are three recognised pathways of complement activation, those being the classical, alternative and lectin-binding pathways, which are activated in different

ways (Figure 1.2). Each pathway utilizes different proteins to recognize the activators and to initiate the cascade. However, all three pathways converge at the level of C3 and share a common terminal pathway. In general, the components of the classical pathway and the terminal pathway are designated with the letter C followed by a number such as C4, whereas the components of the alternative pathway are called “factors” and are identified with a single letter such as factor D. The split products of the components that are generated by proteolytic cleavage are distinguished from their precursors by suffix letters such as C4b. The classical pathway is activated by antigen-antibody complexes. The alternative pathway is constitutively active and directly initiated by surface molecules containing carbohydrates and lipids (Guo & Ward 2005). The lectin-binding pathway is triggered by the binding of either mannose-binding lectin protein (MBL) or ficolin to bacterial/fungal carbohydrate structures, resulting in activation of MBL-associated serine proteases (MASPS), with subsequent engagement of complement proteins, such as C2 and C4 (Guo & Ward 2005).

Traditionally, asthma was considered a simple type I hypersensitivity reaction. Although the complement system forms the central core of innate immunity and has long been appreciated for its proinflammatory properties, it had not been considered to contribute to the pathogenesis of allergic asthma (Baelder *et al.* 2005). However, accumulated data from animal models of allergic asthma and from human asthmatics have fuelled new interest in the role of complement in the allergic response (Baelder *et al.* 2005).

Complement activation has been implicated in the pathogenesis of many inflammatory and immunological diseases. As for asthma, many data suggest a critical role for the anaphylatoxins C3a and C5a, cleavage products of C3 and C5, in the development of the allergic phenotype (Baelder *et al.* 2005).

Complement may participate in susceptibility to asthma because of an intrinsic abnormality in complement activation and generation of C3a, C5a, or other products that affect cellular responses, resulting in Th2 predominance and asthma susceptibility (Wust *et al.* 2006).

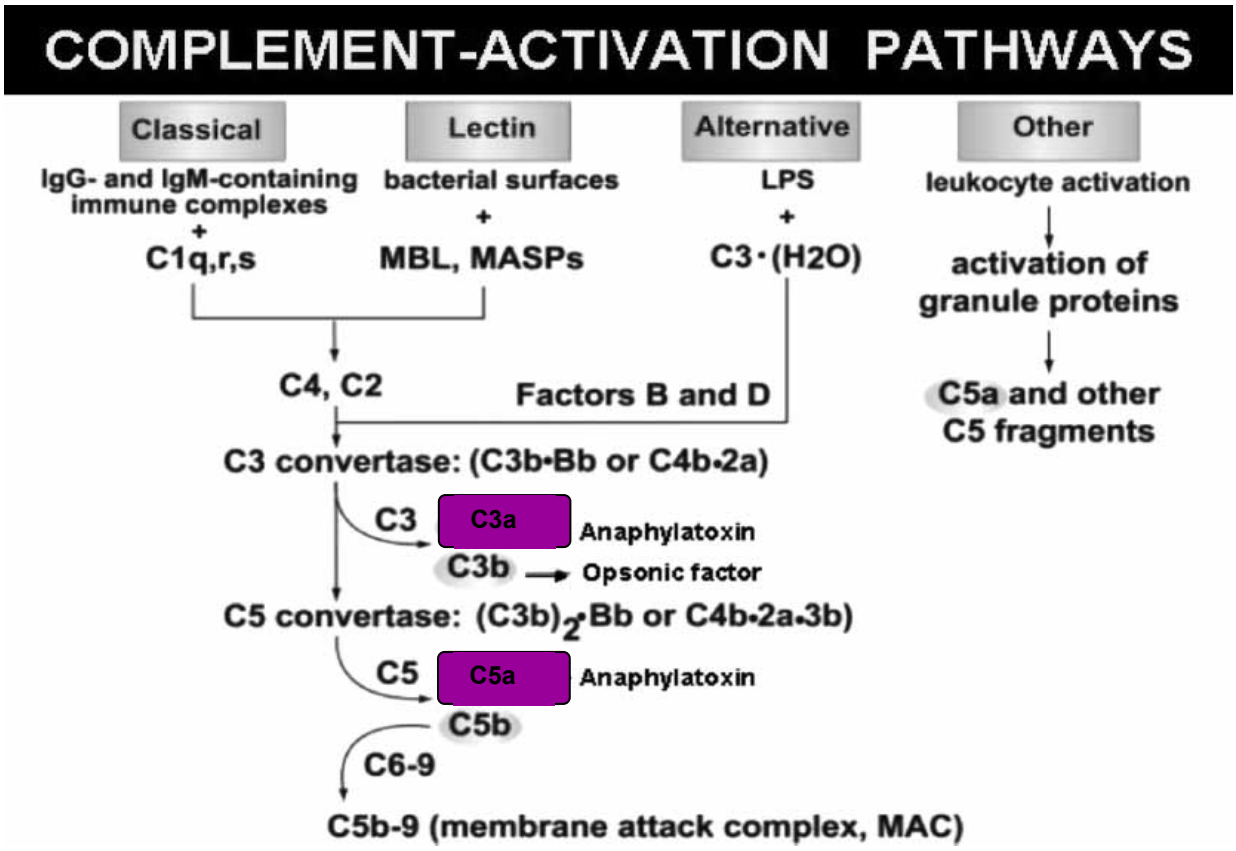

Figure 1.2 The complement system. Activation occurs via three different pathways: classical, lectin and alternative pathways. In addition other proteases are able to cleave complement components to generate anaphylatoxins. Figure adapted from (Sarma, Huber-Lang, & Ward 2006).

1.2.1 The third complement protein (C3)

The most abundant complement protein in the plasma is C3, the third protein of the complement system, which is produced and secreted mainly by hepatocytes but is also synthesized outside the liver. C3 has a basal plasma concentration of 1 mg/ml in humans which increases during inflammation (Kushner *et al.* 1972). It is composed of an alpha and a beta chain. The alpha and beta chains are attached to each other through a disulphide bond (Fong *et al.* 1990). C3 is cleaved upon complement activation with generation of C3a and C3b. Activation of the complement system causes the formation of the enzymatic complex C3-convertase. The C3-convertase cleaves the C3 alpha chain and generates C3a; a small soluble anaphylatoxic fragment that stimulates leukocyte recruitment and activation to release pro-inflammatory cytokines (Hugli 1990). The larger fragment of the C3 molecule, C3b contains a thiolester bond which when exposed binds to target structures in the surrounding area and triggers the terminal part of the complement cascade (Sim *et al.* 1981).

1.2.2 The fifth complement protein (C5)

C5 is a similar protein to C3, being a D-globulin with a molecular weight of 190,000 daltons, and consisting of two polypeptide chains linked by sulphide bridges and non-covalent bonds. C5a is a 12,000 molecular weight peptide derived from the chain of C5, and has anaphylatoxic and chemotactic properties (Monk *et al.* 2007). C5a has been purified and sequenced. Human C5a is composed of 74 amino acids which account for a molecular weight of 8,200 and a single complex oligosaccharide unit attached to the asparagine residue at position 64 accounts for the remainder of the molecular weight (Monk *et al.* 2007). The N-linked carbohydrate moiety of asparagine is not essential for

biological activity but very likely regulates C5a activity *in vivo*. Numerous similarities between the primary structure of C5a and C3a point to their common genetic ancestry. The anaphylatoxic activity is completely, and the chemotactic activity partly lost following removal of the C-terminal arginine residue by the plasma enzyme carboxypeptidase B (also known as anaphylatoxin inactivator) (Monk *et al.* 2007).

Although C3a and C5a share many similar functions, they seem to have to some extent opposing effects on the regulation of adaptive immunity. Thus, C5a is chemoattractant for T lymphocytes (Tsuji *et al.* 2000), potentiates T cell proliferation, and drives the type 1 helper T cell (Th1)-type immune response (Morgan *et al.* 1983). In contrast, C3a has been shown to mediate immunosuppressive effects (Morgan *et al.* 1983) and also to drive the Th2-type immune response (Hawlisch *et al.* 2004).

1.2.3 Complement Receptors

Studies suggest the involvement of C3a and C5a and their cell surface G protein-coupled (Thangam *et al.* 2005) receptors C3aR and C5aR in the pathogenesis of asthma (Fregonese *et al.* 2005). Anaphylatoxins act on their target cells by binding to and activating highly ligand-specific membrane receptors (C3aR and C5aR) belonging to the family of the seven transmembrane domain G-protein-coupled receptors. The expression of these receptors is greatly increased during inflammation. C5aR is expressed on both myeloid cells and nonmyeloid cells including endothelial cells. Each receptor is selective for its respective anaphylatoxin, with no apparent cross-reactivity (Anthony 2007).

Fregonese et al found the receptors to be expressed in the endothelium and smooth muscle cells in the lungs (Fregonese *et al.* 2005), whilst Thangam et al observed that while C3aR and C5aR were expressed in human mast cells, they were not present in cultured primary human or murine ASM cells. Furthermore, C3aR could not be detected in smooth muscle-positive cells of human trachea or bronchus (Thangam *et al.* 2005).

Traditionally, C3aR and C5aR were thought to be present only on myeloid cells such as macrophages, neutrophils and mast cells. However, some studies have also demonstrated these receptors on nonmyeloid tissue cells (Drouin *et al.* 2001b). C3aR and C5aR expression could be detected in alveolar epithelial cells, bronchial epithelial and smooth muscle cells, vascular smooth muscle, and endothelial cells in humans and mice (Tschernig *et al.* 2007). Therefore the receptors are present on many cell types associated with asthma.

C3a and C5a may regulate AHR in asthma via the activation of their receptors in ASM cells (Thangam *et al.* 2005). The rising paradigm exhibits that C3a production at the airway surface serves as a common pathway for the induction of AHR to a range of asthma triggers such as allergens, viral infections, particulate matter, ozone, smoke. On the other hand C5a plays a dual immunoregulatory role by protecting against Th2-mediated immune responses during initiation of responses, and a proinflammatory role after immune responses are established (Wills-Karp & Koehl 2005).

1.2.4 Anaphylatoxins & Asthma

Anaphylatoxins can be defined as the pro-inflammatory complement activation fragments, which are generated in the lungs of both asthmatics and healthy

individuals. The proteolytic cleavage of C4 results in the generation of C4a, which has been shown to possess only weak activity. The anaphylatoxins C3a and C5a are classically seen as proinflammatory mediators of allergic asthma that recruit inflammatory cells, induce oedema, and also cause bronchoconstriction (Lambrecht 2006).

The anaphylatoxins C3a and C5a are released as by-products or split products of complement activation and modulate innate immunity. Besides the two pro-inflammatory anaphylatoxins, the membrane attack complex (MAC) is also generated. These activation products appear to be responsible for promoting and perpetuating inflammatory reactions despite not being the initiating factors in the inflammatory disorders (Guo & Ward 2005). C3a and C5a are responsible for recruiting and activating leukocytes, particularly phagocytic cells such as granulocytes and monocytes and macrophages (Tschernig *et al.* 2007). These anaphylatoxins perform their function by engaging specific receptors on target cells.

C3a mediates an inflammatory response through cell activation to induce, for example, chemotaxis and histamine release. C3a may influence the inflammatory response through its effects on cellular activation and chemotaxis. It is a chemotactic molecule for eosinophils and mast cells and stimulates release of histamine from mast cells as well as basophils (Drouin *et al.* 2002) (Table 1.1).

The C5 protein is cleaved downstream of C3 into C5a and C5b, which forms the first part of the complement MAC. The C5 gene was identified as a susceptibility locus for human allergen-induced asthma (Karp *et al.* 2000). Certain C5 alleles have been found to be associated with protection from the development of both

adult and childhood asthma. C5aR targeting in an established allergic environment reduces airway inflammation and AHR, suggesting a beneficial effect of C5aR blockade in asthma (K+Âhl *et al.* 2006). The contribution of C5 to other major biological hallmarks of asthma (besides enhanced AHR in mice) has not been evaluated (Drouin *et al.* 2006).

Like C3a, C5a mediates an inflammatory response through cell activation to induce chemotaxis and histamine release. C5a also regulates T cell responses (Lalli *et al.* 2008); T cells have a crucial role in asthma and different subsets are involved in orchestrating inflammation (Lalli *et al.* 2008).

Through its chemotactic and cell activation properties, C5a has been implicated in regulating the downstream inflammatory cascade which results in a massive migration of inflammatory cells into the bronchial airway lumen that triggers the release of multiple harmful inflammatory mediators (Peng *et al.* 2005). Also to note, like C5a, C5b-9 also induces potent biological responses, including anaphylatoxic responses, inflammation, tissue injury, and cell lysis at very low concentrations (Peng *et al.* 2005). The role of activated C5 components in sustaining an ongoing airway response to allergen challenge has previously been demonstrated (Peng *et al.* 2005).

The release of C3a and C5a anaphylatoxins contributes significantly to inflammation. C3a and C5a are specifically released into challenged asthmatic lungs, and the inflammatory infiltrate of eosinophils and neutrophils correlates highly with the local production of anaphylatoxins (K+Âhl *et al.* 2006).

Both anaphylatoxins are potent mediators of inflammation, but they also exert immunomodulatory effects on both innate and adaptive immune response. Thus,

C3a and C5a are chemotactic for monocytes and mast cells, cause respiratory burst and secretion of proinflammatory cytokines from macrophages, and trigger mast cell degranulation (Ellati, Dahinden, & Church 1994). Furthermore, C5a has been shown to cause upregulation of adhesion molecules on endothelial cells (Albrecht *et al.* 2004), and both C3a and C5a have been shown to induce expression of proinflammatory cytokines in cultured human umbilical vein endothelial cells (HUVEC) (Monsinjon *et al.* 2003).

Effects of C3a and C5a on inflammatory cells in asthma	
Inflammatory cells	Effects
Mast cells	• Potent activator-degranulation • Secretes mediators: preformed-histamine, newly synthesized lipid- PGs (PGD_2) and LTs (C4), cytokines, growth factors and chemokines • Rich source of proteases
Basophils	• Potent activator-degranulation • Produce mediators including histamine and (PAF)
Eosinophils	• Potent activator • Produce mediators including platelet-activating factor • Major source of cys-LTs
Macrophages	• Potent activator • Produce mediators including PAF
Neutrophils	• Stimulating effector functions • Activation and chemotaxis • Produce lipids (PAF, LTA4), cytokines (TNF-α TGF-β, IL-6), proteases (elastase, collagenase), reactive oxygen species (superoxide)

Table 1.1 Effects of C3a and C5a on inflammatory cells in asthma. PG, prostaglandin; LT, leukotriene; PAF, platelet-activating factor; cys-LT, cysteinyl leukotriene; TNF, tumour necrosis factor; TGF, tumour growth factor; IL-6, interleukin 6 (Information adapted from (Wills-Karp & Koehl 2005).

1.2.5 Complement in animal studies

Animal models have been useful in testing hypotheses of asthma pathogenesis. Studies in murine models have suggested the involvement of C3a and C5a in the development of allergic asthma (Taube *et al.* 2003). Taube et al found the inhibition of complement activation decreases airway inflammation and hyperresponsiveness in mice (Taube *et al.* 2003).

Reports from some laboratories including those of Drouin et al have documented the importance of complement activation, the C3a anaphylatoxin, and its receptor, C3aR, in promoting Th2 effector functions in a mouse model of bronchopulmonary allergy (Drouin *et al.* 2006). Drouin et al found that all features of the experimental allergic asthma model are either abrogated or severely reduced in the genetic absence of the C3aR (Drouin *et al.* 2002). These results suggest that ligands of C3aR may make a major contribution to allergic lung disease.

Drouin and coworkers showed C3aR-deficient mice were protected against AHR, and showed decreased Th2-cell responses, including reduced IL-4 production (Drouin *et al.* 2001a). Further still, in another study involving challenge of antigen-sensitised animals, C3 deficient mice showed diminished AHR, lung eosinophil infiltration and IL-4 production as well as reduced Ag-specific IgE and IgG1 responses, clearly demonstrating that these features of asthma are significantly attenuated in the absence of C3 (Drouin *et al.* 2002). These data provide compelling evidence that C3a and C3aR have the propensity to mediate much of the pulmonary inflammation observed in the acute sensitisation/aerosol situation and suggest that C3a on binding C3aR may

play a significant role in bridging innate and adaptive Th2 immune responses in asthma (Drouin *et al.* 2002).

The role of C5 in asthma remains controversial. Peng et al examined the contribution of C5 in mice at three critical checkpoints during the course of disease- those being a) initiation of airway inflammation, b) maintenance of AHR, and c) sustainment of an ongoing airway response to allergen provocation (Peng *et al.* 2005). Their results indicate that C5 is probably activated intrapulmonarily after infections or exposure to allergen and that C5 inhibition has profound effects at all three critical checkpoints (Peng *et al.* 2005).

Drouin et al's data suggest that C5a plays an important protective role in allergic lung disease by suppressing inflammatory responses and Th2 effector functions observed in their experimental model. There is a suggestion that C5a may play a significant role in tempering inflammation in Th2-driven diseases such as asthma (Drouin *et al.* 2006). Mice lacking C5aR have a reduced ability to clear an infection and to mount virus-specific CD8+ T cell responses. Also, C5-deficient animals have exacerbated Th2-cell responses in an experimental allergic asthma model (Hawlisch *et al.* 2005).

1.2.6 Complement in human studies

There are reports of exaggerated complement production in the lungs of asthmatics which support a causal role for altered anaphylatoxin production in human disease (Wust *et al.* 2006).

Many studies have reported elevated C3a and C5a concentrations in bronchoalveolar lavage (BAL) fluid or plasma in subjects with asthma (Tschernig

et al. 2007). C3a and C5a were reported to be elevated in the BAL of individuals with asthma compared with healthy control subjects at baseline (Nakano *et al.* 2003). Following allergen challenge, C3a is generated in the lung of subjects with asthma but not healthy subjects (Nakano *et al.* 2003;Krug *et al.* 2001). Krug and colleagues demonstrated elevated levels of both C3a and C5a in BAL following segmental allergen challenge of individuals with asthma, whereas no elevations were observed in normal individuals (Krug *et al.* 2001). The role of C5a, however, may be diverse in allergic disease since it was noted to exert anti-inflammatory effects as well (Tschernig *et al.* 2007). This evidence is largely circumstantial, showing that C3a and C5a are there but not what they do, hence making it necessary to investigate what the anaphylatoxins might do in asthma.

1.3 Fibroblast Growth Factors

Fibroblast Growth Factors (FGFs) have been discovered across many species from nematodes to man; they were originally isolated from the brain and pituitary gland (Itoh 2007). The FGF family contains at least 23 members, being one of the largest families of growth factors, and its members are named FGF1 to FGF23**.** These growth factors are recognised for their functions in lung organogenesis and involvement in homeostasis and regenerative processes in adult tissues (Marek *et al.* 2009). FGF proteins have been shown to induce effects on mesodermal and neuroectdermal cells and play a role in angiogenesis, inflammation, wound repair, cell growth, tissue patterns, embryo development, metabolic regulation, cell migration, tissue repair, and maintenance (Nugent & Iozzo 2000). FGF1-FGF23 have been identified in humans and mice (Itoh 2007). Most FGFs are secreted proteins with cleavable N-terminal signal peptides (Itoh 2007). These FGF genes are expressed predominantly during embryonic development and are also expressed in

restricted adult tissues, indicating their potential roles in development (Itoh 2007). FGFs may well play a pivotal role in regulating airway wall remodelling. A number of studies have demonstrated that various FGFs contribute to chronic inflammatory and tissue repair processes as well as to fibrosis in chronic airway diseases such as asthma (Itoh 2007). The genes for FGF1 and FGF2 are widely expressed in developing and adult tissues. The genes for FGF3-FGF6 were identified as oncogenes, whilst FGF7, FGF8, and FGF9 proteins were isolated as growth factors for keratinocytes, an androgen-induced growth factor from carcinoma cells, and a glia-activating factor from glioma cells, respectively (Itoh 2007). FGF10 plays a role in stimulation of the migration and proliferation of keratinocytes (Yun *et al.* 2010). Smallwood et al isolated or identified FGF11-FGF14 genes from the retina by using a combination of random complementary (c)DNA sequencing, homology-based searches of nucleotide sequence databases, and homology-based PCR (Smallwood *et al.* 1996). FGF1, FGF2, and FGF11-FGF14, however, are not secreted proteins, and also do not have N-terminal hydrophobic sequences (Smallwood *et al.* 1996). Although FGF11-FGF14 are not secreted, but function within cells (Itoh 2007), they bear strong sequence similarity to other FGFs. However, the biochemical and functional properties of FGF11-FGF14 are largely unrelated to those of other FGFs (Itoh 2007). FGF11-FGF14 function within cells in an FGFR-independent manner (Itoh 2007).

FGF1 and FGF2 might be released from damaged cells or by an exocytotic mechanism that is independent of the endoplasmic reticulum-Golgi pathway. By phylogenetic analysis, the human FGF gene family can be divided into seven subfamilies (Figure 1.3): FGF1, FGF4, FGF7, FGF8, FGF9, FGF11, and FGF19 (Itoh 2007). Members of the same FGF subfamily have essentially similar specificity (Itoh 2007).

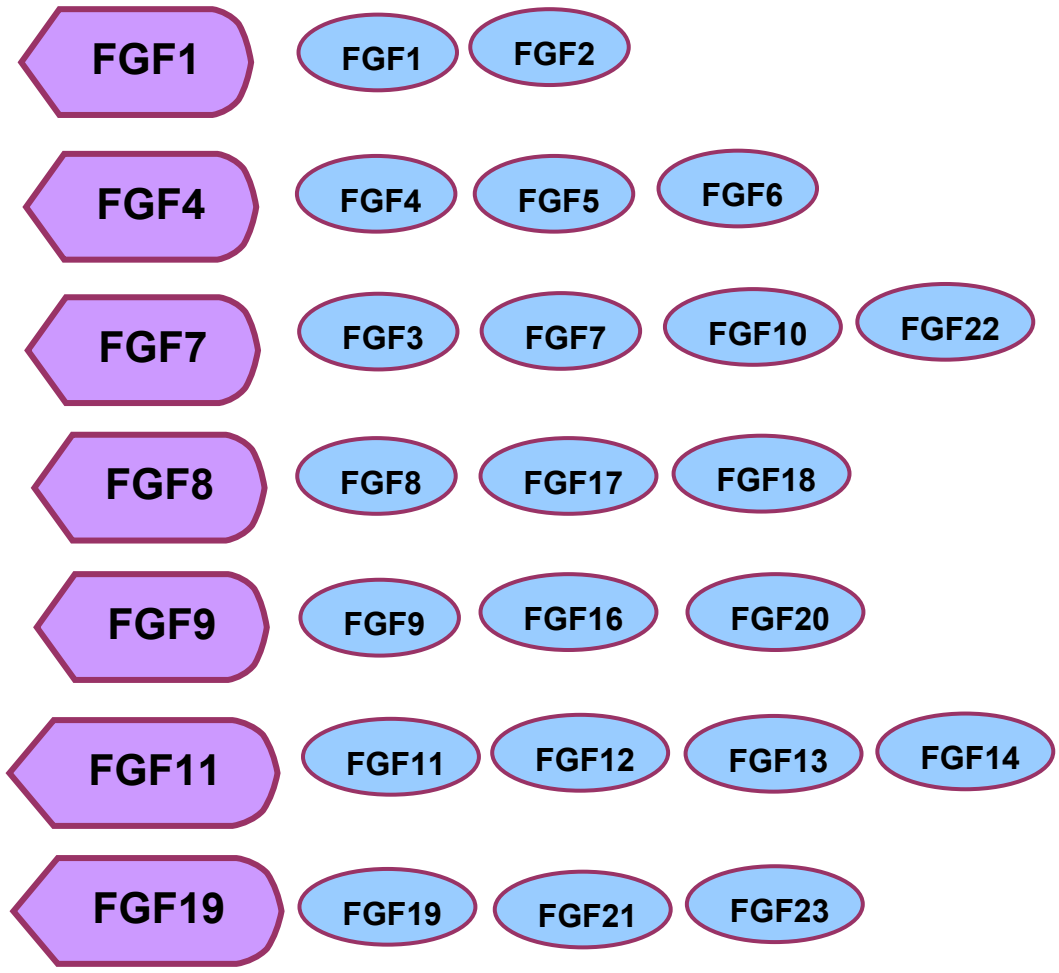

Figure 1.3 FGF family members subdivided into seven subfamilies.

1.3.1 FGF Receptors

Most FGF signalling is mediated by activation of FGF receptors (FGFR) proteins (Itoh 2007). FGFRs belong to the family of receptor tyrosine kinases (RTKs).The FGFs exert their function through interactions between heparin-like proteoglycans and receptors (Itoh 2007). They require the glycosaminoglycan (GAG) side chains of heparan sulphate proteoglycans (HSPG) for high affinity binding to their specific receptors (Marek *et al.* 2009). Four high-affinity receptors have been identified for the FGF family, in humans and mice, named FGFR1 to FGFR4. These receptors are type I transmembrane proteins with an extracellular ligand-binding domain with three immunoglobulin domains (I, II, and III), a transmembrane domain, and a split intracellular tyrosine kinase domain (Itoh 2007).

FGF proteins bind to FGFR proteins and induce their dimerization and the phosphorylation of specific cytoplasmic tyrosine residues (Itoh 2007). The phosphorylation of FGFRs triggers activation of cytoplasmic signal transduction pathways. This activation results in the activation of several signal transduction pathways eliciting different cell responses (Itoh 2007). Phylogenetic analysis indicates potential evolutionary relationships in the gene family. Co-evolution has permitted the evolution of increased ligand-receptor specificity, enabling the formation of preferred ligand-receptor interactions (Itoh 2007). In addition, the alternative splicing of FGFRs has increased their functional diversity. Several alternative splicing events take place following transcription of FGFR1, FGFR2 and FGFR3 and give rise to different isoforms with distinct functional properties (Marek *et al.* 2009). Of particular importance to FGF binding specificity is the third Ig loop, the N-terminal half of which is encoded by an invariant IIIa exon with alternative usage of IIIb or IIIc exons for the C-terminal half (Mohammadi,

Olsen, & Ibrahimi 2005). The immunoglobulin-like domain III is an essential determinant of ligand-binding specificity (Itoh 2007). As a general rule, FGFRs encoding exon IIIb (FGFR IIIb) are expressed on epithelial cells, whereas the FGFRs encoding exon IIIc (FGFR IIIc) are expressed on mesenchymal cells (Mohammadi, Olsen, & Ibrahimi 2005). By contrast, the ligands for FGFR IIIb are often expressed in mesenchymal cells, whereas ligands for FGFR IIIc are expressed in epithelial cells (Marek *et al.* 2009;Itoh 2007). This establishes a paracrine mechanism of signalling between epithelia and mesenchyme that is critical to normal development and tissue homeostasis (Marek *et al.* 2009).

1.3.2 FGF2

The most studied member of the FGF family in asthma pathophysiology is FGF2, also known as basic FGF. This growth factor was first identified in bovine pituitary extracts and, together with FGF1, was named based on its capacity to stimulate the proliferation of the fibroblastic cell line NIH3T3 (Thomas 1987). FGF2 was previously known as basic FGF (bFGF) owing to its high isoelectric point (pI = 9.6), which results from its large number of basic residues. FGF2 is an 18 kD signal protein that varies little between organisms, typically exhibiting sequence homology in excess of 90 % (Nugent & Iozzo 2000). Concentrations of FGF2, a potent fibroblast mitogen, have been reported to be increased in BAL fluid in patients with mild asthma at baseline and further increased in the airways of individuals with allergic asthma after endobronchial allergen challenge (Redington *et al.* 2001;Shute *et al.* 2004).

In view of the potentially important role for FGF2 in the pathogenesis of tissue remodelling in asthma, Shute et al used an immunohistochemical approach to compare the distribution of FGF2 and HSPG in bronchial tissue from patients with mild asthma and normal control subjects (Shute *et al.* 2004). Intracellular

and extracellular FGF2 immunoreactivity was detected in bronchial tissue from control and asthmatic subjects. Intracellular FGF2 was observed within bronchial epithelial cells and in cells within the subepithelial region (Shute *et al.* 2004). Extracellular FGF2 was seen in the pericellular matrix of endothelial cells and in the epithelial basement membrane. Quantitative analysis indicated a greater area of epithelial FGF2 immunostaining in asthmatic tissue than in control tissue (Shute *et al.* 2004). FGF2 is a mitogen for many cells including fibroblasts and endothelial cells- cell types that express CD34 and these cells may therefore be both a source and a target of FGF2 activity. FGF2 release is likely to contribute to the structural changes seen in the asthmatic airway, including fibroblast proliferation and angiogenesis (Shute *et al.* 2004).

FGF2 has been shown to mediate a mitogenic response in mesoderm and neuroectoderm cells such as fibroblasts, osteoblasts, endothelial cells, and smooth muscle cells primarily through transmembrane receptors (Ornitz & Itoh 2001). It is probably best known for its role in the growth and function of vascular cells (Nugent & Iozzo 2000). FGFs have the biological activity of stimulating the proliferation of fibroblasts and angiogenesis, which facilitates potential use in skin wound healing (Yun *et al.* 2010). Both FGF1 and FGF2 are known to be highly released by damaged endothelial cells and macrophages at wound sites, and if FGF2 activity is blocked, wound angiogenesis is almost completely impaired (Yun *et al.* 2010). Sources of FGF2 also include mast cells and T cells. FGF2 is also known to induce scar-free healing (Spyrou & Naylor 2002). Among the FGFs, application studies of wound healing and skin regeneration have primarily been conducted on FGF2 (Yun *et al.* 2010).

1.3.3 FGFR1

FGFR1, also known as basic fibroblast growth factor receptor 1, and CD331, is a receptor tyrosine kinase whose ligands are members of the fibroblast growth factor family. FGFR1 has a molecular weight of 92 kDa and functions to promote mitogenesis in response to fibroblast growth factors. FGFR1 is predominantly expressed in the brain and in mesenchymal tissues in the embryo, in brain, bone, kidney, skin, lung, heart and muscle in the adult, but not in liver (Johnson & Williams 1993). The receptors for FGF1 and FGF2 were identified by cross-linking of the two ligands to the receptors (Itoh *et al.* 1990). The cDNA for the FGF2 receptor was first isolated from a chicken embryo cDNA library (Itoh *et al.* 1990).

1.4 Structural Cells

The ability of structural cells (epithelial cells, fibroblasts, smooth muscle cells and endothelial cells) to synthesise a wide array of inflammatory mediators including proinflammatory cytokines and chemokines implicate these cells as potential inflammatory effector cells in asthmatic airways (Figure 1.4)(Laberge & El Bassam 2004). The exact contribution of specific structural cell types to the overall production of mediators involved in inflammatory cell influx to the airways is unclear (Laberge & El Bassam 2004). Continuation of airway inflammation in asthma likely results from the coordinated expression of several cytokines and chemokines by different cell types working at different time points.

Airways are embedded in the mechanically dynamic environment of the lung. In the adult lung, the mechanical environment is defined by a dynamic balance of surface, tissue, and muscle forces (Niimi 2011). Diseases of the airways modulate both the mechanical stresses to which the airways are exposed as

well as the structure and mechanical behaviour of the airways (Niimi 2011). For instance, in asthma, activation of ASM abruptly changes the airway size and stress state within the airway wall (Tschumperlin & Drazen 2006). The folding of the airway wall into a rosette pattern during bronchoconstriction creates a complex stress field, with the highest stresses compressing the epithelial layer at the inner surface of the airway wall (Niimi 2011). The epithelial cells lining the airway possess the capacity to modulate the inflammatory environment of the airway wall, and produce factors that influence the recruitment, proliferation, and activity of fibroblasts and ASM cells (Tschumperlin & Drazen 2001). Especially, a prominent role has been identified for the epithelium in transducing mechanical stresses (Niimi 2011). A variety of *in vitro* studies have demonstrated that airway epithelial cells, along with lung fibroblasts and ASM cells, are responsive to mechanical stimuli (Niimi 2011). Airway epithelial cells exposed to compressive stresses equivalent to those occurring in the constricted airway increase expression of genes relevant to airway remodelling such as matrix metalloproteinase (MMP)-9, endothelins 1 and 2, TGF-β2, and increase the synthesis of fibronectin, collagen types III and V by cocultured fibroblasts (Tschumperlin & Drazen 2006;Tschumperlin *et al.* 2003). These findings demonstrate that mechanical stress may contribute to the remodelling of the asthmatic airway.

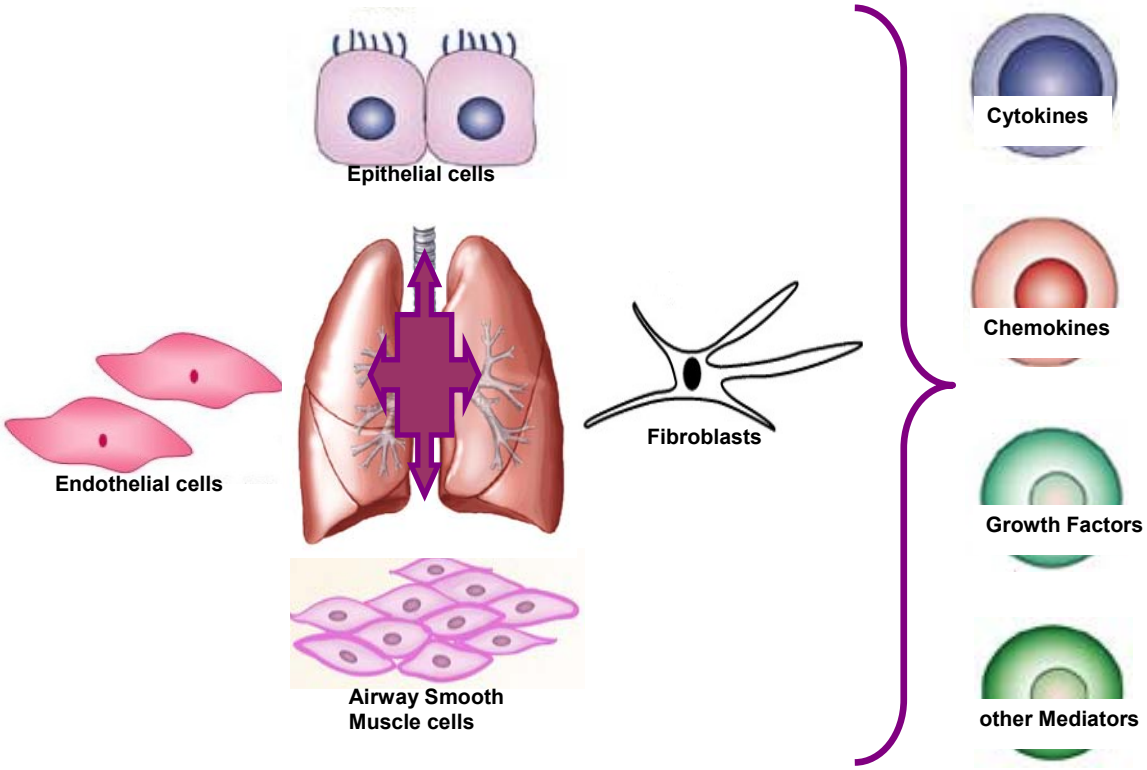

Figure 1.4 Syntheses and secretion of cytokines, chemokines and growth factors by structural cells of the airways.

1.4.1 Airway Epithelial cells

The epithelium of the lung is the first point of contact for inhaled pollutants, airborne allergens, and microorganisms (Bals & Hiemstra 2004). It plays an active role in immunity, through environmental sensing and pathogen detection, signalling to modulate both innate and adaptive immune responses, and direct antimicrobial activities (Kato & Schleimer 2007).

The airway epithelium is pseudostratified, columnar, or cuboidal, and consists mostly of ciliated, undifferentiated, secretory and basal cells (Crystal *et al.* 2008). The airway epithelium lies on a basement membrane, which provides an anchor to facilitate adhesion and migration of epithelial cells, regulates their phenotype and polarity, and separates them from the underlying mesenchymal tissue (Knight & Holgate 2003). Infiltrating inflammatory and immune cells can move freely through this membrane and between epithelial cells (Knight & Holgate 2003).

The airway epithelium plays a vital role in protecting the human body from foreign particles. The tight junctions that fasten together epithelial cells prevent the passage of molecules between adjacent cells, forming a physical barrier that protects underlying tissues (Schneeberger & Lynch 1984). The mucociliary elevator system, consisting of mucus-secreting and ciliated cells, functions to effectively clear inhaled pollutants, allergens and microbes from the lungs (Mall 2008). Recognition of inhaled particles by epithelial cells leads them to secrete mediators such as chemokines and cytokines to attract professional immune cells, but some of their secretions also show direct antimicrobial activity. These include short antimicrobial peptides such as defensins, and larger proteins such as lysozyme and lactoferrin (Hiemstra 2001).

1.4.1.1 Airway epithelium in asthma pathogenesis

A large body of work has focused on immunological aspects of asthma pathogenesis. There is evidence that the epithelium of asthmatics is fundamentally abnormal, with increased susceptibility to environmental injury and impaired repair mechanisms (Kicic *et al.* 2006). Consequently, asthmatic epithelium releases pro-inflammatory mediators as well as growth factors that will act on underlying fibroblasts, driving airway remodelling (Kicic *et al.* 2006). The primary role of the airway epithelium in asthma pathogenesis is supported by the finding that epithelial damage and airway remodelling may predate the onset of asthma symptoms (Busse *et al.* 1999). Furthermore, genetic association studies have identified strong links between asthma and genes expressed by cells of the airway epithelium and underlying mesenchymal tissue (Holgate *et al.* 2007).

It has been suggested that the sub-epithelial fibrosis in asthmatic lungs may contribute to thickening of the airway wall, which in turn may result in physiological alterations such as airflow obstruction and AHR (Cohen *et al.* 2007). Cohen et al evaluated if there are structural and functional differences in the airway epithelium in severe asthma associated with airway remodelling. It was observed that the epithelium was thicker in the subjects with severe, persistent asthma than in those with mild, persistent asthma and normal control subjects (Cohen *et al.* 2007). Epithelial cells, cytokines and growth factors amongst numerous cells and mediators appear to play a part in the remodelling process, but precise roles are yet to be defined clearly (Okayama, Ra, & Saito 2007).

The pulmonary epithelium is the first point of contact for inhaled environmental allergens and is increasingly implicated as a central player in the Th2 cell sensitisation process (Folkerts & Nijkamp 1998). The relationship between the pulmonary innate immune system and surrounding tissue is essential to the proximal events leading to Th2 mediated allergen sensitivity and there is now a growing appreciation of the contribution of innate immune system to asthma (Hammad & Lambrecht 2008). Epithelial cells influence dendritic cell function through direct cell-cell interaction and via the release of mediators (Hammad & Lambrecht 2008). Allergens such as house dust mite can directly or indirectly interact with the innate immune functions of airway epithelial cells (Hammad *et al.* 2009). Many allergens possess epithelial modulatory activity and increase the permeability of this barrier (Kalsheker *et al.* 1996). Indeed, inhaled allergens can directly stimulate epithelial cells to produce a range of mediators such as thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 (Murdoch & Lloyd 2010). These mediators can direct a polarised Th2 cell effector response and further perpetuate the salient features of asthma (Murdoch & Lloyd 2010).

1.4.2 Fibroblasts

Fibroblasts are resident cells of the lungs, which provide structure to the tissue, and also play an active role in inflammation (Spoelstra, Postma, & Kauffman 2001). Although they are regarded as fixed cells of connective tissue origin, they retain the capacity for growth and proliferation and are a pluripotent cell (Bousquet *et al.* 2000).

Fibroblasts are the major type of mesenchymal cell present in the matrix of connective tissue. In addition to their structural role, fibroblasts make important contributions to the inflammatory and remodelling processes by producing

various cytokines, matrix components and matrix-degrading enzymes (Spoelstra, Postma, & Kauffman 2001).

Fibroblasts are able to produce many different chemokines and cytokines upon stimulation. These products can, in turn, activate or attract inflammatory cells, e.g. eosinophils, to the lung (Spoelstra, Postma, & Kauffman 2001). The major feature of lung fibroblasts is their participation in the maintenance of tissue integrity (Laberge & El Bassam 2004). Lung fibroblasts are involved in the repair process in response to inflammation by the release of a variety of extracellular matrix components such as elastin and fibronectin (Laberge & El Bassam 2004). In addition to contributing to airway remodelling, these cells may also play a role in the local regulation of immune and inflammatory responses via the expression of adhesion molecules (intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1)) involved in leukocyte recruitment into lung tissues, leading to the production of a large array of inflammatory cytokines and chemokines (Laberge & El Bassam 2004). Different tissues and even one and the same tissue contain fibroblasts of varying phenotypes (Sime *et al.* 1997). For instance, fibroblast cell lines derived from chronically inflamed tissue and tissues undergoing repair differ in significant ways from those obtained from normal tissues. These differences include the rate of cell proliferation, pattern of gene expression of matrix proteins and integrins, and cytokine production.

1.4.2.1 Fibroblasts in asthma pathogenesis

The remodelling in the airways as seen in asthma is largely a result of altered fibroblast behaviour (Spoelstra, Postma, & Kauffman 2001). Of particular interest in connection with asthma are myofibroblasts, which are contractile, smooth muscle-like fibroblasts often classified on the basis of the filaments they

contain, ie. desmin and alpha-smooth muscle actin (α-SMA) (Powell *et al.* 1999). An increased number of myofibroblasts beneath the reticular basement membrane has been reported in airways of patients with chronic asthma (Kohan, Breuer, & Berkman 2009). Benayoun et al found that the numbers of fibroblasts in the bronchial mucosal subepithelial compartment were increased in severe asthmatics compared with mild asthmatics and controls, leading to the conclusion that fibroblast accumulation is a selective determinant of severe persistent asthma (Benayoun *et al.* 2003). Activated fibroblasts or myofibroblasts may also be involved in the deposition of extracellular matrix and formation of airway fibrosis in asthma (Cai *et al.* 2008).

Fibrocytes are progenitor cells in peripheral blood, which may migrate into the tissue and differentiate into fibroblast-like cells. In mild asthmatics an increased number of fibrocytes in tissue have been correlated with a thicker basement membrane (Nihlberg *et al.* 2006). In response to activation by the pro-inflammatory cytokines IL-1β and TNF-α, fibroblasts synthesise and secrete a wide range of inflammatory mediators, including IL-6, IL-8, TGF-β, and FGFs (Osornio-Vargas *et al.* 1996).

A number of mediators present at sites of inflammation act as chemoattractants for fibroblasts. PDGF and fibronectin have been demonstrated to recruit fibroblasts to inflammatory sites, as well as to activate these cells (Osornio-Vargas *et al.* 1996). The proliferation of fibroblasts is stimulated by a variety of mediators, including cytokines, components of the extracellular matrix and granule proteins of eosinophils and mast cells (Sime *et al.* 1997). Accordingly, FGF, PDGF and TGF-β, which are produced by mesenchymal cells and activated macrophages, have all been shown to act as mitogens for fibroblasts *in vitro* (Osornio-Vargas *et al.* 1996).

Fibroblasts constitute a heterogeneous family of cells that express different phenotypic and functional features depending on their site of origin (Doucet *et al.* 1998a). For example, in human skin fibroblasts IL-4 is thought to increase DNA synthesis, collagen, fibronectin and tenascin deposition (Vita *et al.* 1995). Therefore, it is possible that some of the effects of IL-4 on asthmatic airway hyperresponsiveness can result from a direct and specific action of the cytokine on lung fibroblasts (Doucet *et al.* 1998a).

1.4.3 Airway Smooth Muscle

Smooth muscle cells are multifunctional mesenchymal cells. Changes, such as hypertrophy and hyperplasia in smooth muscle cells are well recognised features of airway remodelling in asthma (Laberge & El Bassam 2004).

Alterations in the contractile and proliferative properties of the smooth muscle cells in airways are associated with several pulmonary disorders (Hirst *et al.* 2004). These cells play the major role in regulating bronchoconstriction and relaxation in asthmatics. In these same patients, these cells proliferate, thereby increasing the total mass of smooth muscle in airway walls (Hirst *et al.* 2004). At the same time, smooth muscle cells are an important source of proinflammatory cytokines, chemokines and other growth factors and, moreover, they produce components of the extracellular matrix, including, MMPs and their inhibitors in tissues. Smooth muscle cells also express adhesion molecules and integrins, which act as receptors for most extracellular matrix proteins (Hirst *et al.* 2004).

1.4.3.1 Airway Smooth Muscle in asthma pathogenesis

Huber and Koesler were the first to describe an increase in ASM tissue in the airways of asthmatics (Huber & Koessler 1922). Airway smooth muscle occupies

a greater role in asthma patients than healthy individuals (Zou *et al.* 2008) and is the most significant cellular change contributing to airway wall remodelling (Lambert *et al.* 1993). Increased smooth muscle mass in asthma may be caused by the proliferative and growth inducing effects of cytokines (Laberge & El Bassam 2004). Multiple other mechanisms may contribute; including constitutional increases from birth caused by genetic, early life and environmental influences and decreased apoptosis/prolonged cell survival (Bai & Knight 2005).

Animal studies suggest that repeated chronic allergen exposure, causing airways inflammation can increase smooth muscle mass along with other features of airway remodelling (Leigh *et al.* 2004). On the other hand, once established, persistent AHR is likely to depend on the persistence of increased ASM mass which has the potential to dominate the mechanical response of the remodelled asthmatic airway (Oliver *et al.* 2007), rather than the persistence of inflammation. It could therefore be argued that regulation of this remodelling is the most important therapeutic target especially for treatment of chronic, severe asthma.

Pepe et al assessed airway remodelling by determining the area and integrity of the epithelium, the smooth muscle mass, and the distance between the epithelium and ASM on bronchial biopsy tissues (Pepe *et al.* 2005). It was found that ASM area was greater in subjects with severe asthma than in subjects with moderate asthma, and also the distance between the epithelial and ASM layers was less in the severe group than in the moderate group (Pepe *et al.* 2005). They came to a conclusion that smooth muscle alteration is the key structural change that distinguishes severe asthma from moderate asthma. This altered structure might explain why patients with severe asthma are clinically more

symptomatic when compared with subjects with moderate asthma (Pepe *et al.* 2005).

1.4.4 Endothelial cells

Common to all vessels of the vasculature is the endothelium, which is a single layered sheet of squamous, polarised cells that are primarily responsible for all signalling and transportation from blood to tissue and vice versa (Cines *et al.* 1998).

Different types of endothelial cells have been isolated and cultured to understand the biology and pathobiology of the vasculature and the angiogenic response (Boisen *et al.* 2010). The cells most widely used in *in vitro* studies are isolated from human umbilical vein, HUVEC, which show a characteristic cobblestone shape when cultured. HUVEC are the most extensively studied endothelial cell type, primarily because they are relatively easy to isolate and culture (Boisen *et al.* 2010). In vivo, angiogenesis is mediated by the microvasculature and the angiogenic response of human microvascular endothelial cells (HMVEC), which has been suggested to be stronger than that of HUVEC because of differing expression of matrix metalloproteases (Jackson & Nguyen 1997). However, HMVEC are generally more fastidious in their culture requirements than HUVEC making long-term culture more challenging (Boisen *et al.* 2010).

Functional studies of endothelial cells have been carried out in culture, as endothelial cells cultured in the presence of an ECM organise themselves into tube-like structures (Boisen *et al.* 2010). This response is seen as a mimic of natural endothelial behaviour during angiogenesis, and as such has been used

as a simple in vitro angiogenesis assay (Boisen *et al.* 2010). As expected for endothelial cells, low passage HUVEC form tube-like structures when cultured on top of gels formed by ECM proteins such as collagen-1 or Matrigel (Unger *et al.* 2002). However, such studies have not been reported for microvascular endothelial cells, although these cells are directly involved in angiogenesis (Boisen *et al.* 2010). While endothelial cells *in vivo* are normally quiescent, cultured endothelial cells are maintained in an artificially activated state characterised by extensive proliferation (Laberge & El Bassam 2004). Airway endothelial cells are a source of chemokines that may contribute to the recruitment and the activation of basophils, eosinophils and lymphocytes into the airways (Laberge & El Bassam 2004).

1.4.4.1 Endothelial cells in asthma pathogenesis

Vascularity is an important component of the remodelling process in airway disease and increased vascularity, angiogenesis, is likely to occur in response to chronic inflammation (Li & Wilson 1997). Increased angiogenesis in the airways of asthmatics is well reported and is now considered to be another of the major components of airway remodelling in asthma (Li & Wilson 1997). Stimulation of angiogenesis is performed by various angiogenic proteins, including several growth factors. The list of pro-angiogenic cytokines and growth factors is extensive and these factors are secreted by inflammatory cells (mast cells and macrophages), smooth muscle and fibroblasts. The principal mediators implicated in angiogenesis and thus increased in asthma are vascular endothelial growth factor (VEGF), TNF-α and FGF2 (Damore 1992).

In healthy individuals, up to 10% of a bronchial biopsy section may consist of vessels with over 500 vessels/mm^2, whereas in an asthmatic biopsy up to 17% of the area may be vascular with over 700 vessels/mm^2 (Li & Wilson 1997).

Wilson *et al.* suggested that the angiogenic process in asthma could be due to recurrent inflammatory episodes, or a response to the two- to threefold increase in tissue volume that occurs in the airways due to remodelling (Wilson & Stewart 1999). Blood vessel size has also been shown to be larger in the asthmatic airway (Li & Wilson 1997). Cross-sectional measurements of blood vessels showed that over 19% of blood vessels in the asthmatic airway to have an area greater than 300μm^2, compared to 12% in non-asthmatic airways (Li & Wilson 1997).

1.5 Th2 cytokines

1.5.1 IL-4

IL-4 is a 15-kd monomer (129 amino acids) produced by Th2 cells, basophils, mast cells, and eosinophils (Akdis *et al.* 2011). IL-4 is a major stimulus for Th2-cell development; it also suppresses Th1-cell development and induces IgE class switching in B cells (Kuhn, Rajewsky, & Muller 1991). A pleiotropic cytokine, IL-4 regulates allergic conditions (Akdis *et al.* 2011).

IL-4 increases the expression of class II MHC molecules in B cells, upregulates B-cell receptors, increases expression of CD23, prolongs lifespans of T and B cells in culture, and mediates tissue adhesion and inflammation. There are 2 types of IL-4 receptors (IL-4Rs). Type I IL-4R binds only IL-4 and consists of 2 receptor chains: IL-4Rα (CD124) and the common γc (CD132). Type II IL-4R binds IL-4 and IL-13 and consists of the IL-4Rα and the IL-13Rα1 chains (Wang *et al.* 2009). IL-4 and IL-4Rα knockout mice have defects in Th2-cell differentiation and reduced serum levels of IgG1 and IgE (Kuhn, Rajewsky, & Muller 1991).

Antibody to IL-4 initially appeared to be a promising strategy for treatment of asthma. In preclinical testing, monoclonal anti-IL-4 blocked development of specific IgE and AHR to ovalbumin in mice, but did not abrogate eosinophilia (Corry *et al.* 1996). Newer agents such as Pitrakinra, a mutant protein which blocks IL-4 and IL-13 receptors, are showing promise for asthma treatment (Antoniu 2010).

1.5.2 IL-13

IL-13 is a 4-helix bundle protein expressed by activated Th2 cells, mast cells, basophils, eosinophils, and natural killer T cells (Akdis *et al.* 2011). IL-13 shares many biological activities with IL-4 (de Vries 1998). This is due to the fact that IL-13- and IL-4-receptor complexes share the IL-4Rα-chain, which is important for signal transduction (de Vries 1998). Its receptors are IL-13Rα1 and IL-13Rα2, and signalling occurs via the IL-4R complex type II, which consists of IL-4Rα and IL- 13Rα1 (Akdis *et al.* 2011). IL-13 activates many of the same signal transduction pathways as IL-4 and induces IgE production. It also activates and recruits mast cells and eosinophils and promotes their survival.

In single-nucleotide polymorphism studies, a combination of relevant polymorphisms in the IL-4 and IL-13 signalling pathways has been associated with a 16.8-fold risk of asthma. Polymorphisms in IL-13 pathways alone were associated with increased risk of asthma exacerbations in children and elevated blood total IgE and eosinophils (Hunninghake *et al.* 2007). In animal “asthma” models, IL-13 knockout mice produce less IL-4, IL-5, IL-10, and IgE and fail to develop goblet cell hyperplasia (McKenzie *et al.* 1998). IL-13Rα1 knockout mice also failed to develop features of asthma and airway remodelling (Akdis *et al.* 2011).

Pulmonary delivery of IL-13 to mice (Grunig *et al.* 1998) or targeted overexpression of IL-13 in the lung induce multiple correlates of asthma pathology, including airway eosinophilia, mucus cell metaplasia, airway fibrosis, eotaxin production and AHR (Zhu *et al.* 1999). Despite their shared activities, studies in animal models have pointed to a preferential role for IL-13 over IL-4 in driving asthma pathology (Grunig *et al.* 1998). In an inducible model of transgenic lung IL-13 expression, fibrosis was initiated upon IL-13 induction and persisted even following withdrawal of the cytokine, demonstrating that IL-13 has the capacity to drive irreversible remodelling processes in the asthmatic lung (Fulkerson *et al.* 2006).

As T cells do not express functional IL-13 receptors, IL-13, in contrast to IL-4, fails to induce Th2-cell differentiation, which is one of the hallmarks of the allergic response. However, IL-13 is required for optimal induction of IgE synthesis, especially in situations in which IL-4 production is low or absent (de Vries 1998). On the other hand, IL-13 inhibits proinflammatory cytokine and chemokine production *in vitro* and has potent anti-inflammatory activities *in vivo* (de Vries 1998).

Both IL-4 and IL-13 can mediate direct activation of lung fibroblasts *in vitro*, inducing eotaxin release, TGF-β production, and collagen gene expression (Doucet *et al.* 1998b).

In summary, it is clear that airways remodelling is an infinitely complex process. Although at least partly inflammation driven, it may also partly be driven simply by mechanical stress on the airways and may be responsible for lasting airways dysfunction even if inflammation is controlled. Structural cells of the airways,

including epithelial, endothelial, smooth muscle and fibroblast/mesenchymal cells are likely involved in remodelling and interact in complex ways through contact and the production of growth and other mediators.

Anaphylatoxins have also been implicated, at least circumstantially, in asthma pathogenesis and it is now clear that structural airways cells, as well as inflammatory cells express their receptors, raising the possibility that they are involved in the remodelling process. The overall aim of the studies described in this thesis is to probe this question by investigating effects of anaphylatoxins on structural airways cells which may be relevant to remodelling.

1.6 Aims and Hypotheses

A considerable amount of literature has been published on asthma and the airways. It is only during the past 20 years or so, however, that information has become available on the impact of the complement system on asthma. Numerous studies have attempted to explain the role of complement components in the asthmatic airway using patients as well as animal models. So far, however, these attempts to establish a link between asthma and anaphylatoxins are somewhat controversial, circumstantial and inconclusive.

The objectives of the studies presented here aim to examine the expression of certain components of the complement pathway and its receptors in the asthmatic bronchial epithelium and on structural cells of the airways, investigate the effects of anaphylatoxins on structural cells of the airways which may be relevant to remodelling, and compare these with the effects of established remodelling mediators, in particular the cytokines IL-4 and IL-13.

It was hypothesised that:

- There is elevated expression/deposition of the complement fragments C3, C3d and C5b-9 in the bronchial mucosa of asthmatics compared to controls
- There is elevated expression/deposition of the complement fragments C3, C3d and C5b-9 in the bronchial mucosa of atopic asthmatics following bronchial allergen challenge
- C3a and C5a play a role in effecting airway remodelling by acting on structural cells, including epithelial cells, endothelial cells, fibroblasts and

smooth muscle cells to induce remodelling changes or production of remodelling mediators.

The specific aims of this thesis were to:

1) Measure expression/deposition of C3, C3d and C5b-9 fragments in the bronchial epithelium, submucosa, ASM and glands of asthmatics & controls

2) Detect expression of C3aR and C5aR in the bronchial epithelium, submucosa, ASM and glands of asthmatics & controls, and in addition to study the effects of allergen challenge of atopic asthmatics

3) Detect expression of C3aR and C5aR on structural cells of the airway involved in remodelling propagated *in vitro*

4) Investigate C3a and C5a-stimulated production of remodelling mediators (FGFs, IL-8) by these cells at the mRNA and protein level

5) Assess biological functions of C3a and C5a on those structural cell types which may be relevant to remodelling (i.e. by proliferation assays)

This would allow for a better insight into the role of anaphylatoxins, their receptors and remodelling growth factors in human asthma.

Chapter 2: Materials & Methods

2.1 Cell culture

2.1.1 Epithelial cell culture

2.1.1.1 A549

A549 cells (adenocarcinomic human alveolar basal epithelial cells) were obtained from the American Type Culture Collection. Cells were expanded in 75 cm^2 tissue culture flasks (Thermo Fisher Scientific, UK) containing 15 ml Dulbecco's Modified Eagle Medium ((DMEM), Invitrogen) supplemented with 10 % foetal bovine serum ((FBS), Invitrogen) and 1 % penicillin-streptomycin solution (Invitrogen). Cell cultures were incubated at 37 ^{o}C in a humidified incubator with 5 % CO_2 in air. Fresh medium was added after every two days of culturing. Cells were routinely subcultured or passaged (following trypsinisation; see section 2.1.5) after reaching confluence. Cultures of reasonable confluency, around 80 %, were used for all studies. A549 cells used were all between passages 2-3.

2.1.1.2 BEAS-2B

BEAS-2B (American Type Culture Collection) cells were cultured in the same way as A549 cells (see section 2.1.1.1). BEAS-2B cells are adenovirus-transformed cells established from normal human bronchial epithelium.

2.1.1.3 HBEpC

Human Bronchial Epithelial Cells (HBEpC) were purchased from Promocell. These cells are isolated from the surface epithelium of human bronchi and stain positively for cytokeratin. HBEpC were grown in 75 cm^2 tissue culture flasks

(Thermo Fisher Scientific) pre-coated with collagen I (5 mg/ml; Invitrogen) in ascetic acid (0.02 M) by culturing for at least 1 hour at 37 ^{o}C, with 5 % CO_2 in a humidified incubator. The cells were cultured in Airway Epithelial Cell Growth Medium Kit, which consists of Basal Medium and a Supplement Pack. The growth medium was formulated to consist of 2 % serum and 1 % antibiotics/antimycotics. Fresh medium was added after every two days of culturing. Cells were routinely subcultured (following trypsinisation; see section 2.1.5) after reaching confluence, and cultures of reasonable confluency, around 80 %, were used for all studies. HBEpC used were between passages 2-4.

2.1.2 Fibroblast cell culture

2.1.2.1 MRC-5

Human lung fibroblasts (MRC-5, American Type Culture Collection), a transformed line, were grown and cultured exactly like A549 cells (see section 2.1.1.1).

2.1.2.2 HPFC

Human Pulmonary Fibroblast Cells (HPFC) which are isolated from human lung tissue were purchased from Promocell. According to Promocell the HPFC's have been tested as fibroblast specific surface antigen positive. They are often used for studying lung tissue repair, tissue remodelling after injury, or the response after pulmonary inflammation. The HPFC 75 cm^2 tissue culture flasks and 12-well plates (Thermo Fisher Scientific) were pre-coated with collagen I (5 mg/ml; Invitrogen) in acetic acid (0.02 M) by culturing for at least 1 hour at 37 ^{o}C, with 5 % CO_2 in a humidified incubator for better cell adherence and cell

growth. HPFC were grown and cultured just like MRC-5 and A549 cells (see section 2.1.1). Fresh medium was added after every two days of culturing. Cells were routinely subcultured or passaged (following trypsinisation; see section 2.1.5) after reaching confluence, and cultures of reasonable confluency, around 80 %, were used for all studies. Fibroblast cells used were at passage 2. Primary cultures of human lung fibroblasts maintain their phenotypic characteristics *in vitro* throughout a substantial number of passages.

2.1.3 Smooth muscle cell culture

2.1.3.1 HBSMC

Human Bronchial Smooth Muscle Cells (HBSMC; also referred to as HSMC) were purchased from Promocell and grown and cultured in 75 cm^2 tissue culture flasks (Thermo Fisher Scientific) with Smooth Muscle Basal Medium (Promocell) formulated to contain 2 % v/v serum (FCS). Fresh medium was added after every two days of culturing. Cells were routinely subcultured (following trypsinisation; see section 2.1.5) after reaching confluence, and cultures of reasonable confluency, around 80 %, were used for all studies. Smooth muscle cells used were between passages 2-3. HBSMC are isolated from the bronchi of single donors and stain positive for smooth muscle α-actin. These cells are suitable for *in vitro* studies of asthma and other pulmonary diseases. According to Promocell, HBSMC have been tested smooth muscle α-actin positive, von Willebrand factor (vWF) negative and CD90 negative.

2.1.4 Endothelial cell culture

2.1.4.1 HUVEC

Human umbilical vein endothelial cells (HUVEC) obtained from Promocell were cultured in Endothelial Cell Basal Medium (Promocell) supplemented with Endothelial Growth Medium singlequots (Promocell) and FBS (2 % final concentration). To promote HUVEC attachment and growth, all culture surfaces were pre-coated with 5 mg/ml collagen I (Invitrogen) in acetic acid (0.02 M) by culturing for at least 1 hour at 37 °C with 5 % CO_2 in humidified air. Fresh medium was added after every two days of culturing. Cells were routinely subcultured by trypsinisation (see section 2.1.5), and cultures of around 70-80 % confluency were used for all studies. HUVEC cells used were all between passages 2-3.

2.1.4.2 HPMEC

Human Pulmonary Microvascular Endothelial Cells (HPMEC) were purchased from Promocell. HPMEC were cultured in Endothelial Cell Growth Medium (Promocell), which is a low serum medium optimized for the cultivation of endothelial cells from blood vessels. This medium was chosen as it does not contain VEGF. Generally, VEGF leads to higher endothelial cell proliferation in culture. It also has multiple effects on the cell metabolism, which may interfere with certain experimental setups. HPMEC were cultured in 75 cm^2 tissue culture flasks pre-coated with 1 % gelatin (Sigma-Aldrich, St. Louis, MO). The cells were grown in an atmosphere of 5 % CO_2 at 37 °C and routinely subcultured by trypsinisation (see section 2.1.5) after they reached confluence. The cells were allowed to grow till they reached 80 % confluence before treatment. HPMEC cells used were all between passages 3-5.

Batches of HPMEC are isolated from the lung of a single donor. According to the manufacturer the cells are routinely analyzed by immunofluorescent staining: they stain positive for CD31 and vWF, and negative for smooth muscle α-actin. Because of their location at the interface between the circulating fluid in the lumen and the surrounding tissue, microvascular endothelial cells are highly active and closely involved in numerous physiological processes. The lung has a vast endothelial surface area, which is essential for the exchange of gases. HPMEC are most appropriate for studying human lung diseases.

2.1.5 Cell subculture

Cells were subcultured by trypsinisation. Cells were passaged such that the monolayers never exceeded 75-90 % confluency. Growth medium was aspirated from the culture flasks. Cells were washed in Hanks balanced salt solution ((HBSS), Invitrogen), followed by incubation with 0.05 % trypsin supplemented with 0.53 mM ethylenediaminetetraacetic acid solution salt ((EDTA.4Na): (1X Trypsin-EDTA), Invitrogen) for 3-5 minutes. Detachment of cells was confirmed by microscopy. In the case of A549 and MRC-5 cells trypsinisation was stopped by the addition of 2 times the volume of growth medium (supplemented DMEM, Invitrogen); for HBEpC, HSMC and the endothelial cells, trypsinisation was stopped by the addition of an equal volume of Trypsin Inhibitor (Invitrogen). The cells were transferred to a 50 ml Falcon tube and centrifuged at 200 x g for 5 minutes and resuspended in 1 ml of their respective growth medium. Cell viability was examined by exclusion of Trypan Blue (Sigma-Aldrich) in a haemocytometer visualised by light microscopy for manual cell counting.

2.1.6 Cell starvation

For gene expression and enzyme-linked immunosorbent assay (ELISA) experiments cells were subcultured and seeded on 12 well plates (Thermo Fisher Scientific) at a density of 0.1 x 10^4cells/ml in a total volume of 1 ml per well. For western blot experiments cells were seeded on 6 well plates (Thermo Fisher Scientific) at a density of 2.5 x 10^4cells/ml in a total volume of 1.5 ml per well.

Prior to treatment, the cells were maintained in serum free medium for 24 hours. A549 and MRC-5 cells were maintained in a 1:1 Nutrient Mixture of Ham's F-12 Medium (Sigma Aldrich) and DMEM (Invitrogen) supplemented with 1% penicillin-streptomycin solution (Invitrogen). HBSMC, HBEpC, HPMEC, HPFC and HUVEC were all maintained in their respective growth medium with reduced serum concentration (0.5% FBS, Promocell).

2.1.7 Cell treatment

Cells were treated with various agents following characterisation of time course and concentration responses. Time points selected for measurement of RNA expression were 4, 8 and 24 hours, while time points tested for protein expression were 24, 48 and 72 hours. The serum-free and low-serum cultures were stimulated with different cytokines: recombinant human TNF-α (R&D Systems, Minneapolis, MN USA) at 10ng/ml, recombinant human IL-4 (R&D Systems, Minneapolis, MN USA) at 10 ng/ml, recombinant human IL-13 (R&D Systems, Minneapolis, MN USA) at 10 ng/ml, and anaphylatoxins: human complement proteins C3a and C5a (Fitzgerald Industries International, Inc) at a concentration range of 10^{-10} M to 10^{-7} M. C3a and C5a proteins are prepared from normal human serum, with the concentration of the proteins in serum being

approximately 1.25 +/- 0.52 mg/ml. According to the manufacturer the products had been tested at serum donor level by FDA approved methods. The time points and concentrations used were based on existing literature. On the last day of culture, the total cell culture lysate and culture supernatant were separately harvested. All culture supernatant samples were centrifuged to remove cellular debris then divided into 200-300 µl aliquots and stored at -20 °C until used. The experiments with each of the structural cell types were repeated three to five times.

2.2 Immunohistochemical Studies

2.2.1 Patients & Study Design

The expression of complement components and FGF2 was investigated in the bronchial mucosa of asthmatics and controls. Bronchial biopsies were obtained at fibreoptic bronchoscopy from controls and asthmatics. Asthma was defined as a history of typical symptoms and ≥ 12% reversibility of the PEF/FEV_1 and/or methacholine PC20 ≤ 8mg/ml in the year prior to the study. Asthmatics were stratified for disease severity. Biopsies were also obtained from mild atopic asthmatics before and 24 h after bronchial allergen challenge with an allergen to which they were sensitised according to a standard protocol (Kariyawasam *et al.* 2007). Patient characteristics are described in Table 2.1. All patients were recruited through the Department of Asthma, Allergy and Respiratory Science at Guy's Hospital. The study was approved by the Guy's (London Bridge) Research Ethics Committee and all subjects provided written, informed consent to participate in the studies and to have their tissues stored anonymously in our HTA licensed tissue bank.

PATIENT CHARACTERISTICS			
Subjects	Sex (F:M)	Age (yr)	FEV_1 (% predicted)
Control (n=12)	4:8	22.5 (19-38)	106.5 (93-118)
Asthma (n=32)			
Moderate/Severe (n=11)	3:8	59 (27-73)	65 (42-77)
Mild (n=21)	11:10	28 (21-44)	101.2 (81.2-133)
Pre Allergen Challenge (n=11)	4:7	28 (22-38)	98.2 (81.2-120.3)
Post Allergen (24h) Challenge			58.9 (41.3-97.1)

Table 2.1 Patient Characteristics. Data are expressed as the median (range). The mild asthmatics prior to challenge (n=11) are included in the total of 21 mild asthmatics.

2.2.2 Biopsy Sample Preparation

Biopsies were OCT processed. For OCT processing, bronchial biopsies were fixed immediately in freshly prepared 4 % paraformaldehyde (PFA; BDH, Poole, UK) in phosphate-buffered saline (PBS), pH 7.4 for 2 hours. 4 % PFA in 0.1 M phosphate buffer is one of the common solutions used for immunohistochemistry. The biopsies were then washed with 15 % sucrose (Sigma Chemical CO, Poole, UK) in PBS, embedded in OCT compound and snap-frozen. Sections were cut from biopsies at a thickness of 6 μm using a

cryostat, further air-dried for 1 hour at 37 °C, wrapped in foil and stored at -80 °C until use.

2.2.3 Complement IHC *ex vivo*

Immunohistochemistry (IHC) was performed on tissue sections. Optimal concentrations of all antibodies used were determined in pilot experiments. IHC staining of biopsies was performed using the peroxidase anti-peroxidase (PAP) method. The PAP method is a further development of the indirect staining method. The indirect technique involves an unlabelled primary antibody (first layer) which reacts with the tissue antigen and a labelled secondary antibody (second layer) which reacts with the primary antibody. The secondary antibody recognises the immunoglobulin of the animal species in which the primary antibody has been raised. This method ensures improved signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody and thus is a more sensitive method. The second layer antibody can be labelled with a fluorescent dye (indirect immunofluorescence) or with an enzyme such as a peroxidase or alkaline phosphatase. The PAP method involves adding a layer which is an antibody to peroxidase, coupled with peroxidase, making a very stable peroxidase anti-peroxidase complex. This complex acts as a third layer antigen, greatly increasing the sensitivity of the method because the peroxidase molecule is not chemically conjugated to the anti-Ig, but immunologically bound and loses none of its activity. It also allows for a much higher dilution of the primary antibody. The peroxidase is then developed by DAB or other substrates to produce a colorimetric end product.

An initial blocking step was performed to block endogenous peroxidase in the tissue using hydrogen peroxide (H_2O_2; 1:30), made up in a 3 molar solution of

sodium nitrite (made in PBS). Bronchial tissue was blocked for 40 minutes and then the slides were washed in PBS twice for 5 minutes each time. The slides were then incubated overnight at room temperature with the control and primary antibodies (Table 2.2), which were made up using 5% normal human serum (in PBS). The slides were washed twice in PBS for 5 minutes each time, and then the monoclonal mouse antibody labelled slides were treated with the second layer rabbit anti-mouse (1:30; DakoCytomation, Inc) in 5 % serum in PBS for 30 minutes whilst the rabbit (anti-C5b-9) slides were treated with the second layer mouse anti-rabbit (1:50; Dako) in 5 % serum in PBS for 30 minutes. After a further wash in PBS for 5 minutes twice, the slides were incubated with the third layer: mouse anti-C3, C3d, C3aR and C5aR slides were treated with mouse PAP (1:50; Jackson ImmunoResearch Laboratories, Inc USA) for 30 minutes, whilst rabbit anti-C5b-9 was treated with rabbit PAP (1:50; Jackson ImmunoResearch Laboratories, Inc USA) for 30 minutes. The PBS washing step was repeated again after which the sections were developed with DAB (Sigma) in the dark for 10-20 minutes. During the developing period the sections were monitored periodically by eye and under a microscope. After sufficient developing the slides were washed in water for 10 minutes and counterstained with Mayer's Haematoxylin (Sigma-Aldrich) for 2 minutes. This was washed off with water. The slides were then air-dried and the sections mounted under coverglasses using DPX (BDH).

Staining and measurement of area were measured objectively using a digital analysis programme (Zeiss KS300 3.0) linked to an Olympus microscope. Sections were observed at x20 magnification. Geometric calibration (in μm) and light were adjusted. Overall expression of complement components followed by their expression on various cells was reported. This was done by outlining the individual tissue cells using the above programme and then subtracting the

stained areas from total areas measured. For example, the epithelium of the bronchial biopsy was drawn around to obtain a total area percentage. The programme determines the positive staining area as a ‘brown’ area percentage from the total area by subtracting it from the total.

PRIMARY ANTIBODIES		
Monoclonal Antibody	**Concentration**	**Source**
Mouse anti-C3	1:50	Santa Cruz Biotech
Mouse anti-C3d	1:25	AbD Serotech
Rabbit anti-C5b-9	1:100	Abcam
Mouse anti-C3aR	1:50	Abcam
Mouse anti-C5aR	1:40	AbD Serotech

Table 2.2 Primary antibodies used for IHC.

2.2.4 FGF2 IHC *ex vivo*

FGF2 immunoreactivity was measured in sections of bronchial biopsies from the same patients (see Table 2.1) according to the protocol described above (section 2.2.3). The primary layer was rabbit polyclonal anti-FGF2 (1:50; Abcam); the secondary layer was mouse anti-rabbit (1:50; Dako) and the tertiary layer was rabbit PAP (1:50; DakoCytomation).

Objective, quantitative measurement of the total stained cells per unit area was performed as before using a digital analysis programme (Zeiss KS300 3.0) linked to an Olympus microscope**.** The numbers of positively stained cells were counted separately in the epithelium and submucosa and their total surface areas in each section were measured under x40 magnification. Results were expressed as the numbers of positive cells per unit area.

2.2.5 ICC *in vitro*

Immunocytochemistry (ICC) was performed on cultured structural cells of various types. Cells were grown as described in the cell culture section 2.1 and seeded at 20,000 cells per chamber in four-chamber slides (VWR International). The cells were allowed to reach confluence after which they were gently washed in PBS and then fixed for 10 minutes in 4 % PFA then washed in PBS twice. The chamber slides were stained with the primary layer, mouse monoclonal C3aR (1:50; Abcam) and mouse monoclonal C5aR (1: 40; Abcam) diluted in 5 % normal human serum in PBS. Isotype-matched control antibodies were used as a negative control in the experiments. Incubation was overnight at room temperature. The slides were washed twice in PBS for 5 minutes each time and then treated with the second layer rabbit anti-mouse (1:30; DakoCytomation) in 5 % serum in PBS for 30 minutes. The slides were washed twice again in PBS for 5 minutes each time, and then treated with the third layer mouse PAP (1:50; Jackson ImmunoResearch Laboratories) for 30 minutes. The PBS washing step was repeated again after which the sections were developed with DAB (Sigma) in the dark for 10-20 minutes; washed with water and then counterstained with Mayer's Haematoxylin (Sigma-Aldrich) and mounted using glycergel. The cells were viewed and photographed with a Zeiss Axioskop 2 microscope equipped with appropriate filters.

2.3 Cell proliferation assay

Cell proliferation was assayed using the CellTiter 96® Aqueous Assay (Promega, USA). Cells were plated out 5000 cells per well in duplicate in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, MA), allowed to adhere, serum starved for 16 hours and then exposed to various mediators: TNF-α (10 ng/ml; R&D Systems), IL-4 (10 ng/ml; R&D Systems), IL-13 (10 ng/ml; R&D

Systems) and C3a and C5a (10^{-9} M to 10^{-7} M; Fitzgerald Industries International) in the respective cell growth medium. Aliquots of cells were exposed for 24 hours, 48 hours and 72 hours, after which a MTS/PMS solution (20 μl) was added to the media wells. The cells were then incubated at 37 °C in a humidified incubator with 5 % CO_2 in air for a further 3.5 hours. Results were read on a microplate reader at the test wavelength of 450 nm. The MTS assay is a colorimetric assay for measuring the activity of enzymes that reduce MTS dye to formazan dyes, giving a purple colour. It allows the assessment of proliferation of cells.

2.4 Gene Expression Studies

2.4.1 Extraction of cellular RNA

Samples of typically 100,000 cells were harvested in buffer RLT. Buffer RLT contains the chaotropic agent guanidine isothiocyanate and the denaturant β-mercaptoethanol (Sigma), and is used to lyse cells or tissue while protecting ribonucleic acid (RNA) via deactivation of RNase enzymes. It preserves RNA for subsequent analysis. Lysed cells were collected from the culture plates using cell scrapers (Thermo Fisher Scientific, Waltham, MA), and collected into 0.5 ml microfuge tubes. All samples were stored at -80 °C until RNA extraction.

All RNA extractions were performed using the RNeasy Mini Kit with QIAshredder (Qiagen, Inc), following the manufacturer's protocol, including the optional 1 minute final spin, eluting RNA in 30 µl RNase-free water. RNA quality and yield were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

2.4.2 Reverse Transcription

Total RNA (500 ng) was reverse-transcribed in a volume of 40 µl (Table 2.3). RNA extracts were first incubated with 0.2 µg Random Hexamer (Bioline) at 70 °C for 5 minutes and allowed to cool down to room temperature. A master mix containing 5X Reaction Buffer (MBI Fermentas), dNTP mix (10 mM; MBI Fermentas), and Ribolock Inhibitor (40 u/µl; Fermentas) was then added and the mixture incubated at room temperature for 5 minutes. Revert-Aid™ Reverse Transcriptase (200 u/reaction; Fermentas Life Sciences) was then added and the reaction mix subsequently incubated using a PTC-200 Thermal Cycler (MJ Research) at room temperature for 10 minutes; 42 °C for 1 hour; and then at 70 °C for 10 minutes to stop the reaction. The reverse transcription reaction products were 10-fold diluted to 400 µl to be within the linear range of PCR amplification.

Reagent	Volume
RNA	500ng
Random Hexamer	8 µl
Deionised water	Upto 25 µl
5X Reaction Buffer	8 µl
dNTP mix	4 µl
Ribolock Inhibitor	0.5 µl
Deionised water	1.5 µl
Revert-Aid™ Reverse Transcriptase	1 µl
Total	40 µl

Table 2.3 Volumes of each reagent added to reaction.

2.4.3 Real-Time Quantitative PCR

Primer sets to examine gene expression by real-time quantitative PCR (qPCR) were purchased from the Gene Expression Inventoried Assays selection (Applied Biosystems) (Table 2.4). Aliquots (2.5 µl) of the diluted cDNAs were subjected to PCR in 15 µl reactions with 2X Taq Master Mix (Applied Biosystems), 18S rRNA (Applied Biosystems), and the specific primer/probe set in 384 well plates. Q-PCR was carried out at 50 °C for 2 minutes, 95 °C for 10 minutes, 95 °C for 15 seconds, and 60 °C for 1 minute for 50 cycles using an ABI Prism 7900HT Thermal Cycler (Applied Biosystems). All messenger (m)RNA species were measured in triplicate samples, repeated at least three times and expressed in relation to the human housekeeping gene, 18S mRNA

levels. Results were analysed using SDS 2.1 Software (Applied Biosystems), and data presented as “Relative Expression.”

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection. The hydrolysis probe (Taqman) technique was used here where the probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.

	Gene	Taqman® Gene Expression Assay ID
Complement Components	C3	Hs00163811_m1
	C5	Hs00156197_m1
	C3aR	Hs00377780_m1
	C5aR	Hs00383718_m1
Fibroblast Growth Factors	FGF1	Hs00265254_m1
	FGF2	Hs00266645_m1
	FGF9	Hs00181829_m1
	FGF11	Hs00182803_m1
	FGF17	Hs00182599_m1
	FGF21	Hs00173927_m1
	FGF22	Hs00221001_m1
	FGFR1	Hs00241111_m1
	FGFR2	Hs01552926_m1
	FGFR4	Hs01106908_m1
	FGFRL1	Hs00222484_m1

Table 2.4 List of TaqMan Gene Expression Assays.

2.5 Protein Expression Studies

2.5.1 BCA Assay

Total protein concentrations in samples were determined using a BCA Protein Assay kit (Thermo Scientific) following the manufacturer's instructions: 10 µl of each standard and unknown samples were pipetted into microplate wells in duplicate. 200 µl of working reagent were added to each well with gentle mixing of the plate. The plate was then covered and incubated at 37 ^{o}C in a humidified incubator with 5 % CO_2 in air for 30 minutes and then cooled to room temperature. The absorbance was measured at or near 562 nm on a plate reader.

2.5.2 Protein extraction from cultured cells

Cells were rinsed in ice cold PBS and lysed in ice cold RIPA buffer (1 % Nonidet P-40, 0.5 % deoxycholate, 0.1 % SDS in PBS) containing a protease inhibitor cocktail (Roche). For a 12-well plate 60-100 µl of buffer were used per well. The cells were incubated with the lysis buffer for 5 minutes on ice and then scraped using a sterile cell scraper (Thermo Fisher Scientific); collected and transferred in a microfuge tube and incubated a further 30 minutes on ice. After that the samples were sonicated for 5-8 seconds and incubated on ice for 30 minutes. Finally, the samples were centrifuged at 12,000 x g for 5 minutes at 4 ^{o}C to pellet the cell debris; the supernatant was carefully collected and stored at -20 ^{o}C until used.

2.5.3 ELISA

Concentrations of IL-6, IL-8, FGF2, thymus- and activation-regulated chemokine (TARC) and VEGF in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (all purchased from PeproTech, London) according to the manufacturer's instructions. The standard curves for human IL-8, VEGF, IL-6 and FGF2 ranged from 16-2000 pg/ml, 16-1000 pg/ml, 32-2000 pg/ml and 63-4000 pg/ml respectively. The individual assays were reported to show no cross-reactivity. A summary of an ELISA protocol is outlined here: A 96-well plate was incubated overnight with the capture antibody at 0.5 µg/ml in PBS (pH 7.20 in sterile water), and subsequently blocked with PBS containing 1 % BSA (R&D Systems). After washing the plate with PBS buffer containing 0.05 % Tween-20 (R&D Systems), appropriate dilutions of samples and standards were added, incubated for 2 hours at room temperature, washed, and incubated with a detection antibody for 2 hours at room temperature. After washing, streptavidin-HRP conjugate (1:200; R&D Systems) was added. Finally, ABTS substrate (100 µl) (R&D Systems) was added. The reaction was stopped with 2 N H_2SO_4 (R&D Systems), 50 µl per well. The plate was read on an ELISA plate-reader at an optical density (OD) of 450 nm with wavelength correction set at 650 nm.

2.5.4 Western blotting

Samples were prepared using RIPA buffer (1 % Nonidet P-40, 0.5 % deoxycholate, 0.1 % SDS in PBS) containing a protease inhibitor cocktail (Roche), and loading buffer (20 % glycerol, 1M Tris HCl pH 6.8, bromophenol blue) to contain an equal amount of protein (20 µg). 15 µl of each sample was loaded and subjected to electrophoresis on 10 % SDS polyacrylamide gels

(Biorad, Hercules, CA, USA). Protein standard markers (Bio-Rad Laboratories) were loaded and run parallel to each blot as an indicator of the molecular weight. The gels were run in running buffer (25 mM Tris, 192 mM glycine, 1 % w/v SDS) at 100 Volts for 1 hour and 20 minutes. The proteins were then transferred onto a 0.45 µm nitrocellulose membrane (Sigma-Aldrich, USA) using transfer buffer (25 mM Tris, 192 mM glycine) at 30 Volts for 1 hour. After transfer, non-specific protein binding was blocked by incubation of the membrane with 5% non-fat dry milk in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1 % Tween-20) for 1 hour at room temperature. This was followed by incubation with primary antibodies: rabbit FGFR1 (1:500; Abgent, Beverly, MA, USA) and β-actin (1:2500; Sigma) in milk/TBS-T overnight at 4 °C with rocking. The immunoblots were then washed 3 times with TBS-T and incubated with a secondary antibody, goat anti-rabbit HRP (1:1000; DakoCytomation) to detect binding of the primary antibody for 1 hour at room temperature with gentle shaking. The immunoblots were developed and visualised using the Enhanced chemiluminescence (ECL) system (Amersham Hyperfilm™ ECL) and a Kodak Image station (Kodak Digital Science). When blots for β-actin were not performed in parallel to the target, the target blots were stripped using Re-Blot Plus Strong Solution (10X; Millipore) and re-probed.

This protocol was also followed for C3 (1:100; Hycult biotech) and C5 (1:100; Hycult biotech) detection. The secondary antibody for both primary antibodies was rabbit anti-mouse HRP (1:1000; DakoCytomation).

2.6 Data and statistical analysis

Results illustrated in the figures were compiled and analysed using Prism. Raw data were used for statistical analysis. Immunohistochemical data were compared using the Mann-Whitney U test and the Wilcoxon test for unpaired

and paired data, respectively. Paired Student's t test was used to compare treated groups with untreated (control) group. For all tests, P values of less than 0.05 were considered significant.

Chapter 3: Bronchial mucosa

3.1 Introduction

Complement activation has been implicated in the pathogenesis of many inflammatory and immunological diseases. Most important biological activities of complement are derived from C3 and C5. The split products of C3 are C3a and C3b. The breakdown of C3b generates C3d, which binds to antigens and enhances their uptake by B cells and dendritic cells. The split products of C5 are C5a and C5b. C5b forms the core of the membrane attack complex, or MAC (C5b-9). This is the final complex of the complement system, and has multiple effects on diverse cells.

Many data suggest a possible role for the anaphylatoxins C3a and C5a in asthma and airway remodelling (Baelder *et al.* 2005). Anaphylatoxins are generated in the lungs of both asthmatics and healthy individuals. They are classically seen as proinflammatory mediators of allergic asthma that elicit an inflammatory response by binding to their respective receptors C3aR and C5aR (Lambrecht 2006). Although the expression of C3aR and C5aR on human bronchial epithelial and smooth muscle cells has previously been described (Drouin *et al.* 2001b), overall the amount of this expression and cellular distribution of these receptors in the human asthmatic bronchial mucosa are unknown. There has also not been any specific study to see whether or not there is concomitant expression of the anaphylatoxin ligands and their receptors in the asthmatic bronchial mucosa.

As seen by most conducted studies and investigations, bronchoscopy and bronchial biopsy are the most commonly used techniques for determining the extent of airway remodelling (Lazaar & Panettieri 2003). However, there are still areas involving asthma and remodelling that are unclear, therefore

understanding the pathogenesis and mechanisms driving airway remodelling could lead to new approaches to therapy and, specifically, retard the progressive decline in lung function that may commence in asthmatics at an early stage (Cohen *et al.* 2007).

In view of this it is hypothesised that:

1. Components of complement are expressed in the bronchial mucosa of asthmatics;
2. Bronchial allergen challenge of atopic asthmatics is associated with elevated expression of complement components and their receptors in the bronchial mucosa;
3. Assess the number of FGF2 positive cells in the bronchial mucosa of asthmatics & controls.

3.2 Results

3.2.1 Complement staining

Cells expressing immunoreactivity for the different complement components and the two receptors stained brown after developing with DAB (Figure 3.1). Omission or substitution of the primary antibody with an isotype matched antibody of the same species was used as a negative control. No immunoreactivity was observed in the negative controls. Greater detail of the staining in individual cell types are shown in Figure 3.2 where expression of C3 has been used as a representative expression. The bronchial biopsy from an asthmatic subject has been used to show an example of how immunoreactivity for a particular component was obtained by outlining the individual cells i.e. the epithelium.

3.2.2 Complement component immunoreactivity in the bronchial mucosa of asthmatics

Global expression of complement components and their receptors was compared in the bronchial mucosa of a group of asthmatics with a range of disease severity and normal controls. The characteristics of the patients are summarised in Chapter 2, Table 2.1. Comparing all asthmatics with controls (Figure 3.3), increased immunoreactivity for C3 and C5b-9 was observed in the epithelium, smooth muscle and submucosa, but not the glandular areas of the bronchial mucosal sections. C3d immunoreactivity was elevated only in the epithelium. Expression of C3a and C5a receptors was detectable on cells in all areas of the mucosa (epithelium, submucosa, smooth muscle and glands) although the amount of expression did not differ in asthmatics and controls

except in the case of the C3aR, expression of which was elevated on smooth muscle.

3.2.3 Effect of allergen bronchial challenge of mild atopic asthmatics

Expression of immunoreactivity for the complement fragments and C3a and C5a receptors was measured in the bronchial mucosa of mild atopic asthmatics before and 24 hours after allergen bronchial challenge. The patients are described in Chapter 2, Table 2.1. Changes in expression of all analytes measured were not marked following challenge, although C3 immunoreactivity was significantly elevated in the bronchial epithelium while C5aR and C3d immunoreactivity were increased in the submucosa (Figure 3.4).

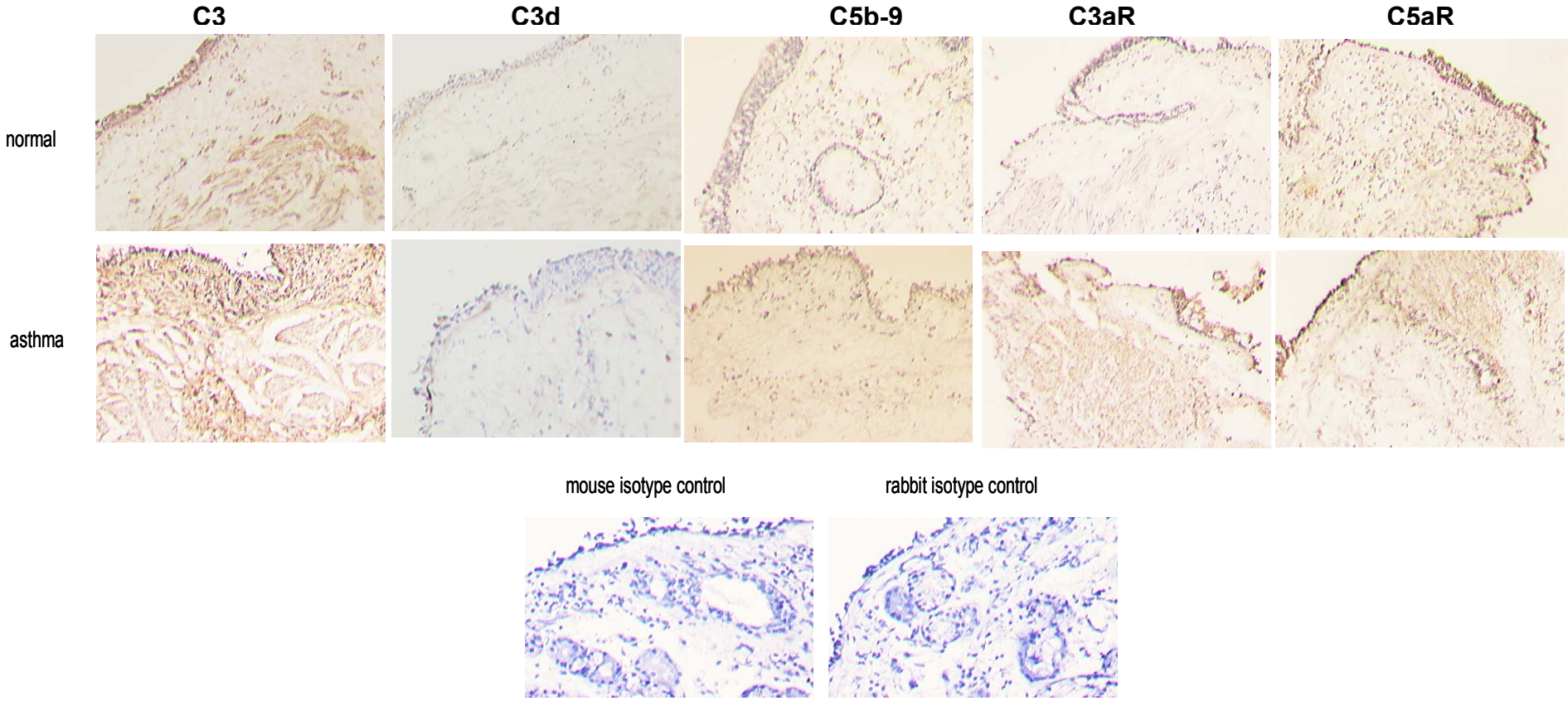

Figure 3.1 Expression of complement components on asthmatic bronchial tissue by single immunohistochemistry. Sections of bronchial biopsies from normal and asthmatic subjects were immunostained for C3, C3d, C5b-9, C3aR and C5aR as described in Chapter 2: Materials & Methods, section 2.2.3. Magnification: x20.

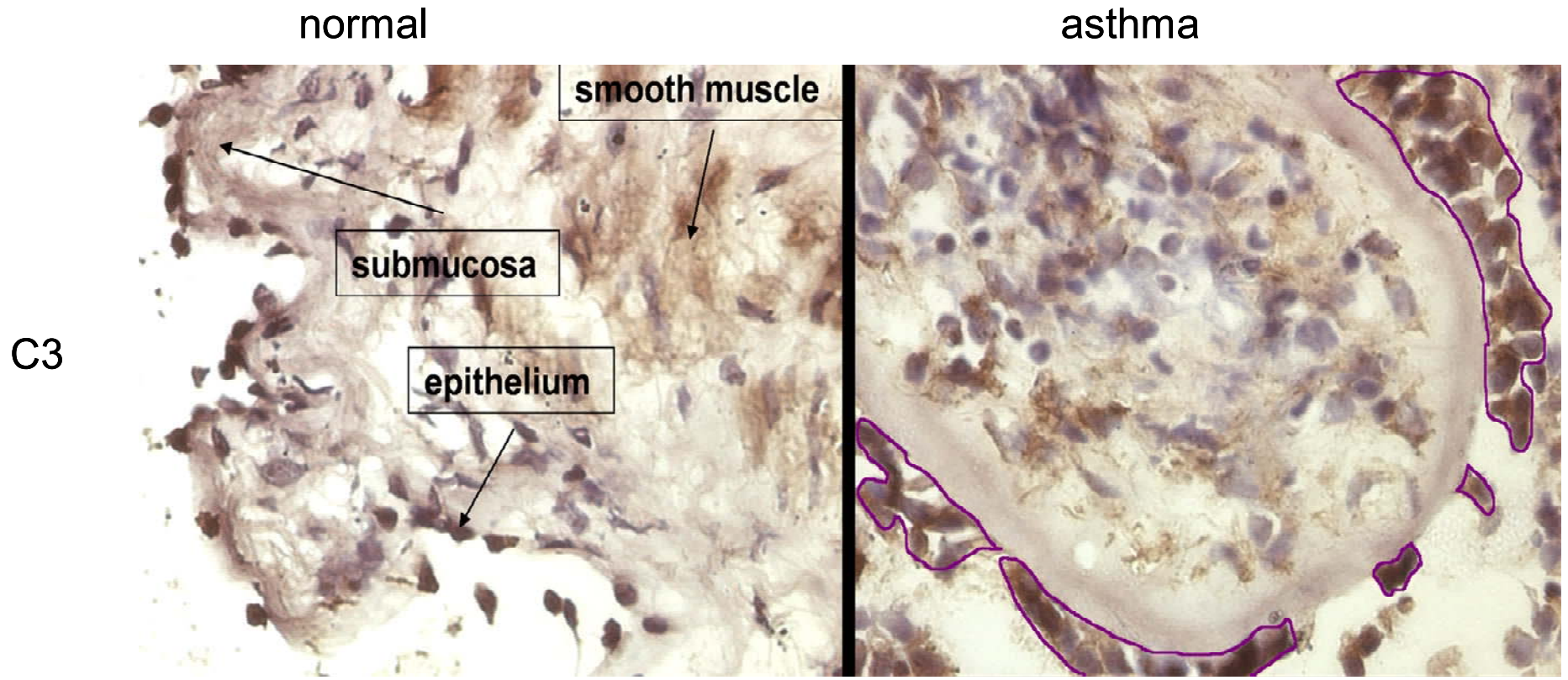

Figure 3.2 Expression of C3 on bronchial tissue by single immunohistochemistry. Purple outline on asthma biopsy shows epithelium. Magnification: x40.

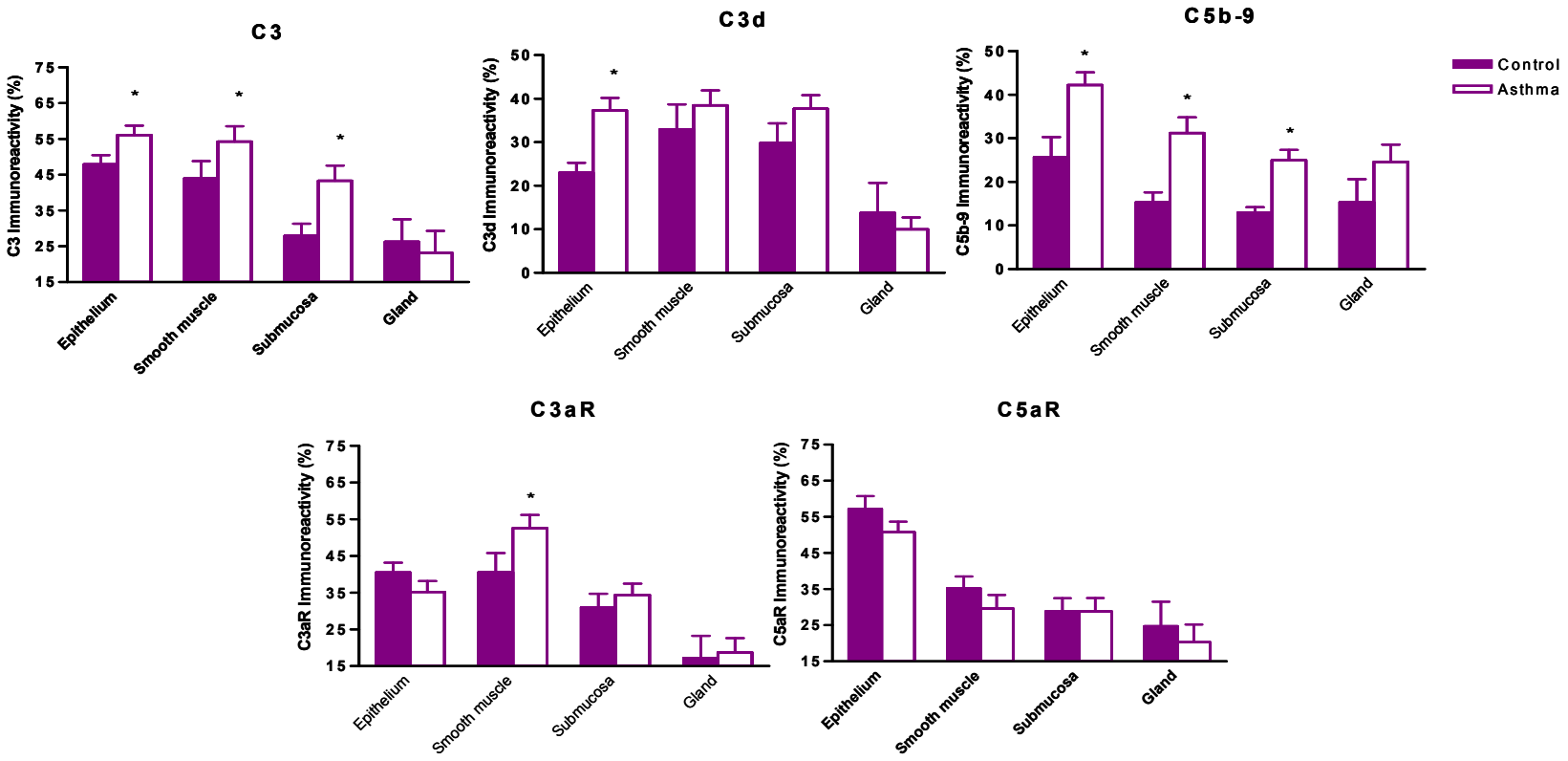

Figure 3.3 Global expression of immunoreactivity for complement fragments and receptors in sections of the bronchial mucosa from asthmatics and controls. Patient characteristics are detailed in Chapter 2, Table 2.1. Figures represent percentages of the total areas of the biopsies stained. Bars show mean and SEM for 12 controls and 32 asthmatics. * $p<0.05$ vs control (Mann-Whitney U test).

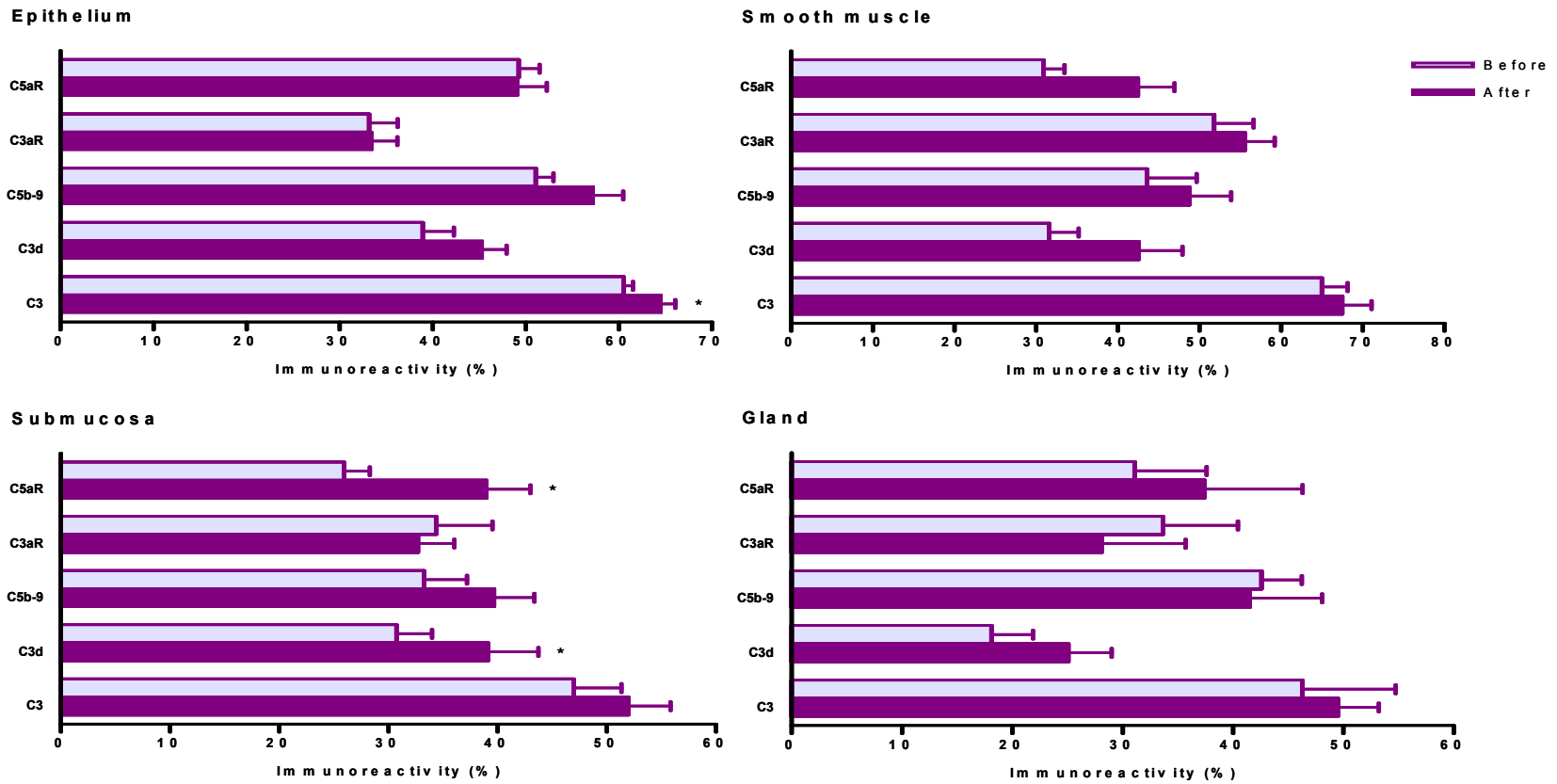

Figure 3.4 Immunoreactivity for complement fragments and receptors in sections of the bronchial mucosa from mild atopic asthmatics before and after allergen challenge. Figures represent percentages of the total areas of the stated sub-structures (epithelium, smooth muscle, submucosa, glandular tissue) stained (n=20). * $p<0.05$ (Wilcoxon matched pairs test).

3.2.4 FGF2 staining

FGF2 immunoreactive cells were measured in sections of bronchial biopsies from a group of asthmatics of a range of disease severity and normal controls (for details see Chapter 2, Table 2.1). Positive cells stained brown after developing with DAB (Figure 3.5). Omission or substitution of the primary antibody with an isotype matched antibody of the same species was used as a negative control. Cells expressing FGF2 immunoreactivity were observed in sections of the bronchial mucosa (total epithelial and submucosal cells) of the asthmatics and controls. The positive cells are shown in higher magnification (x40) in Figure 3.6 in two bronchial biopsies representative of a normal and an asthmatic subject. The median number of $FGF2^{+}$ cells was higher in the asthmatics compared with the controls, a difference largely attributable to increased numbers of these cells in the submucosa rather than the epithelium (Figure 3.7). FGF2 expression did not correlate with that of any of the components of complement or its C3a and C5a receptors (data not shown).

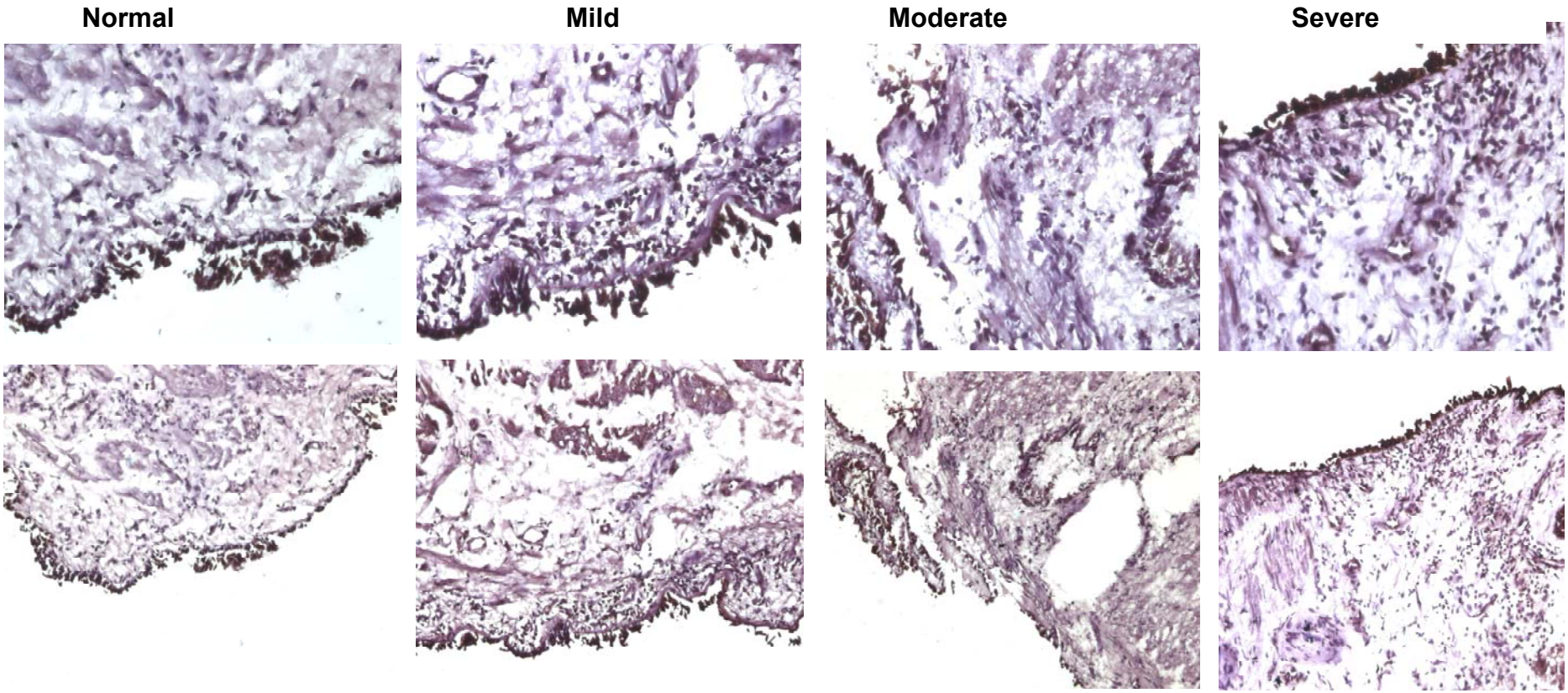

Figure 3.5 Expression of FGF2 on asthmatic bronchial tissue by single immunohistochemistry. Sections of bronchial biopsies from normal, mild, moderate and severe asthmatic subjects were immunostained for the growth factor FGF2 as described in Chapter 2: Materials & Methods, section 2.2.4. Magnification: top panel x20; bottom panel x10.

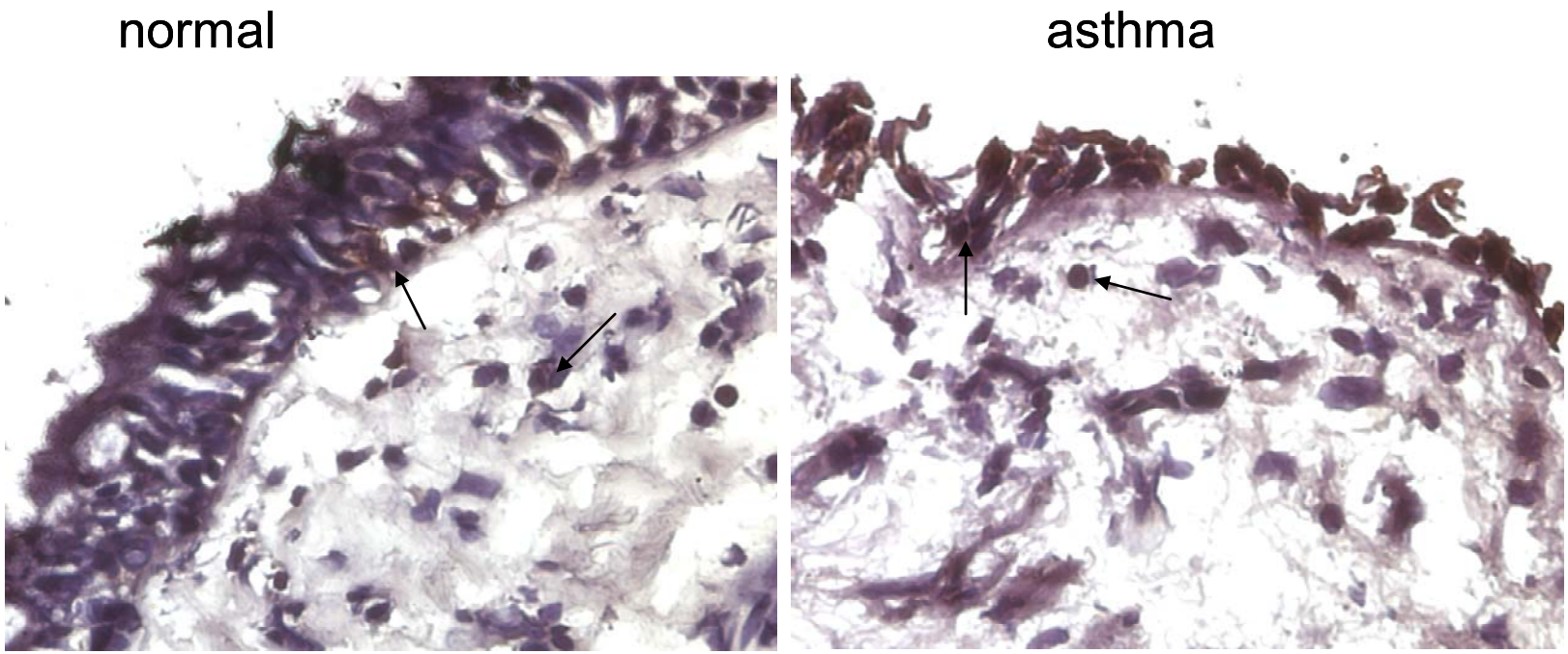

Figure 3.6 Expression of FGF2^{+} cells at high magnification on bronchial tissue by immunohistochemistry. FGF2 positive cells shown with arrows. Magnification: x40.

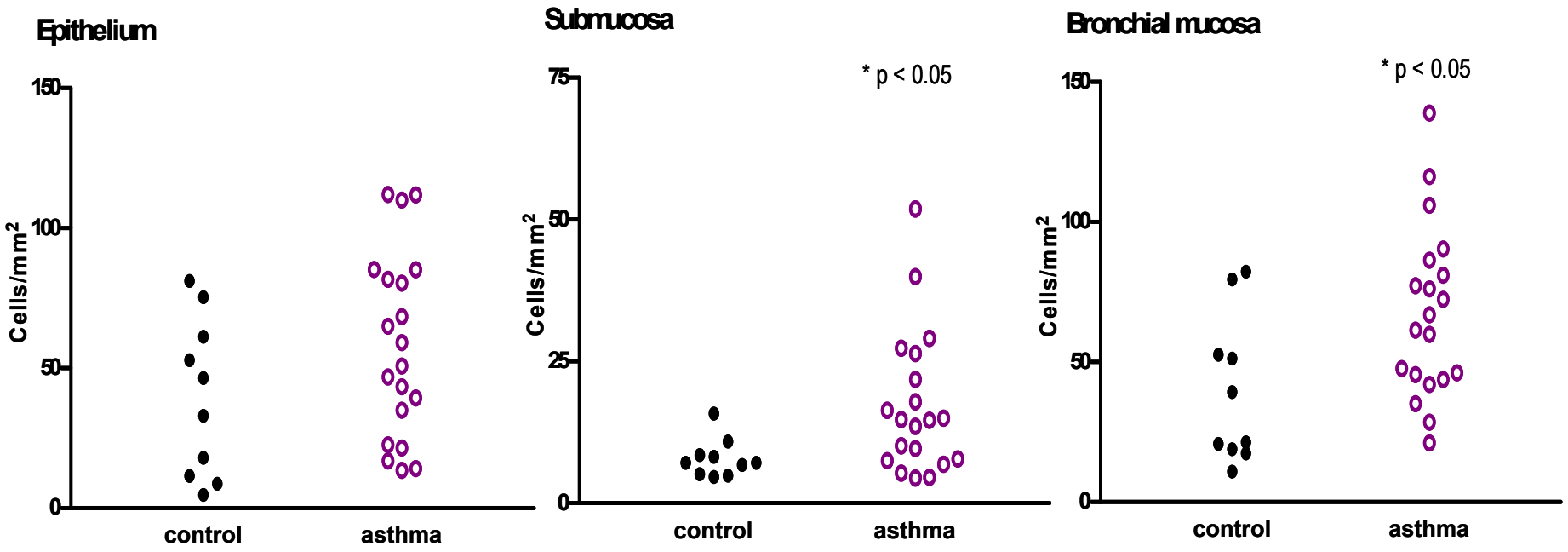

Figure 3.7 Numbers of FGF$^+$ cells in the bronchial mucosa, epithelium and submucosa of control and asthmatic subjects. n=10 control subjects, n=20 asthmatic subjects, p*<0.05 vs control (Mann-Whitney U test).

3.3 Discussion

The hypotheses set out in this chapter were upheld to some extent although the changes observed were very small. Nevertheless, the evaluation of the complement components by IHC as presented in this chapter is novel as the expression of these particular components has not yet been studied in the bronchial mucosa of patients with a range of asthma severity. The most important biological activities of complement are derived from C3 and C5 which give rise to the anaphylatoxins. The expression of C3a and C5a could not be investigated directly in this study because there are no suitable commercially available antibodies. Consequently, in addition to the anaphylatoxin receptors the expression of C3, C3d and C5b-9 were measured. C3 and C3d were used as substitute biomarkers for C3a. C3d, an opsonin, is another smaller cleavage product of C3 and it is therefore not unreasonable to hypothesise that its production may reflect that of C3a, a larger cleavage product. C5b-9 was measured owing to its importance as an end effector following complement activation.

Although there are no studies that have investigated the expression of complement components in the lungs of subjects with asthma, there is one previous study which looked at the expression of the anaphylatoxin receptors in lung tissues. Fregonese and colleagues conducted a study on the expression of C3aR and C5aR in lung tissue from patients who died of fatal asthma and patients who died of nonpulmonary causes (used as controls) (Fregonese *et al.* 2005). They showed elevated expression of both receptors in subjects who died of asthma compared with control subjects and those with mild intermittent asthma (Fregonese *et al.* 2005). The high expression of C3aR and C5aR shown in their study was suggested to be a hallmark of fatal asthma as the intensity of

staining of both receptors was noted to be relatively low in biopsies from patients with mild intermittent asthma although further, more systematic analysis was not pursued. Nevertheless, their data support the hypothesis that complement plays an active role in the pathogenesis of fatal asthma and suggest that the airway and parenchymal vessels and the airway epithelium might be the major sites of action of C3a and C5a during the fatal attacks (Fregonese *et al.* 2005).

Owing to the limited range of suitable antibodies, the data presented here can provide only surrogate evidence for the involvement of complement, particularly C3a and C5a in asthma. It is not possible to determine from these data whether the immunoreactivity reflects deposition of complement fragments or in situ synthesis, or both.

The data provide circumstantial evidence for the involvement of components of the complement cascade in the pathogenesis of asthma in the sense that increased C3 and C5b-9 immunoreactivity was observed in the epithelium, smooth muscle and submucosa, but not the glandular areas of bronchial mucosal sections in asthmatic subjects compared with controls. C3d and C3aR immunoreactivity were likewise elevated although only in the epithelium and smooth muscle respectively. Elevated C5a receptor expression was not observed in any area of the bronchial mucosa of the asthmatics compared with controls. Although epithelial C3aR expression and submucosal C5aR expression correlated inversely with lung function in the asthmatic biopsies, these data are difficult to interpret because overall expression of these receptors was not elevated in these areas in the asthmatics compared with the controls. The data might have been influenced by anti-asthma therapy of the moderate/severe asthmatics. Expression of complement fragments and C3a and C5a receptors in the bronchial mucosa of mild atopic asthmatics showed little

change before and after allergen bronchial challenge. The use of 24-hour post allergen challenge could have been late for the expression of the complement components to be seen.

Although an obvious pattern of deposition and/or expression was not noted for the complement components in the bronchial mucosa, expression of the fragments and receptor immunoreactivity was detectable not only in the submucosa of the sections, where inflammatory cells such as eosinophils which are known to express receptors might have been expected to be situated, but also in the epithelium and smooth muscle, supporting the hypothesis that these structural cells are at least theoretically capable of responding to stimulation by complement fragments.

Our inability to observe elevated FGF2 immunoreactivity in the asthmatic biopsies compared with the controls contrasts with a previous report by Shute et al in which a more detailed quantitative analysis suggested elevated extracellular deposition in the pericellular matrix of both epithelial and endothelial cells (Shute *et al.* 2004).

Chapter 4: Epithelial Cells

4.1 Introduction

The epithelium of the lung represents an immense surface area of interface between the environment and the internal milieu, estimated to approximate that of a tennis field (Zaas & Schwartz 2005). The epithelial cells lining the airways are particularly well adapted to the protection of the airway mucosa from major sources of injury (e.g., tobacco smoke, pollutants, viruses, and bacteria) (Puchelle *et al.* 2006). These cells fulfil a number of critical functions in innate airway defence mechanisms. At the surface of the airway epithelium (bronchi and bronchioles), these specialized cells include columnar ciliated cells, mucous (goblet) cells, Clara cells, and basal cells (Puchelle *et al.* 2006). At the submucosal level, the glands form tubules that feed into a collecting duct and then into a ciliated duct that is continuous with the airway surface. Tubules are lined with mucous cells (proximal region) and serous cells (distal acini). More than 90% of the airway mucus is provided by these glands (Puchelle *et al.* 2006). These airway cells can rapidly change their structure and functions, either to adapt to changes in the local environment or to repair the epithelium after injury. In the upper and lower airways, the surface airway epithelium is normally pseudostratified (Puchelle *et al.* 2006). This implies that all cells are attached to the basement membrane but not all reach the airway lumen. In the proximal bronchioles, the epithelial cells become more cuboidal and, in addition to ciliated cells, contain secretory Clara cells. In the most distal bronchioles, only Clara cells are identified (Puchelle *et al.* 2006).

The airway epithelium participates in innate immune responses (Zaas & Schwartz 2005). This immune dysregulation is partly responsible for asthma pathogenesis, and the airway epithelium has gathered increasing attention as a primary driver of asthma (Holgate 2008). Recognition of pathogens is mediated

by specific binding of conserved pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs). These PRRs are present on the surface of leukocytes and airway epithelial cells, and as part of the surfactant secreted by the epithelium (Zaas & Schwartz 2005).

A large body of work has focussed on immunological aspects of asthma pathogenesis, and the role of a Th2 biased immune response in asthma is well supported (McGee & Agrawal 2006). There is evidence that the epithelium of asthmatics is fundamentally abnormal, with increased susceptibility to environmental injury and impaired repair mechanisms (Kicic *et al.* 2006). Consequently, asthmatic epithelium releases pro-inflammatory mediators as well as growth factors that will act on underlying fibroblasts, driving airway remodelling (Kicic *et al.* 2006).

IL-13 is known to be a central mediator of the allergic asthmatic phenotype, exerting a number of effects on airway epithelial cells (Wills-Karp 2004). It has been shown to play a role in the development of mucous cell hyperplasia (Kuperman *et al.* 2002) and in inducing expression of epithelium-derived growth factors (such as TGF-β) and chemokines (such as eotaxin, monocyte chemoattractant protein (MCP)-3) to name but a few (Booth *et al.* 2007). The released factors, in turn, affect neighbouring epithelial cells as well as other cell types within the airway walls such as fibroblasts and smooth muscle cells as well as inflammatory leukocytes (Davies & Holgate 2002). While it is well documented that epithelial cells, including those of the airways, produce and release growth factors, the mechanism, or mechanisms, regulating cytokine-induced release of growth factors has not been fully elucidated (Booth *et al.* 2007).

There are limited studies exploring complement components in the context of the airway epithelium, together with Th2 cytokines and other remodelling mediators. Here it was hypothesised that complement components particularly the anaphylatoxins are capable of affecting proliferation of airway epithelial cells and their production of remodelling mediators.

The specific aims of the studies described in this chapter were to:

1. Assess the expression of C3aR and C5aR on lung epithelial cells;
2. Assess the proliferative response of epithelial cells to stimulation with anaphylatoxins and Th2 cytokines;
3. Examine C3a and C5a-stimulated production of important remodelling mediators (growth factors) by epithelial cells at the mRNA and protein level, comparing with Th2-type cytokine-stimulated production as a positive control.

4.2 Results

4.2.1 Expression of complement receptors on lung epithelial cells

Immunocytochemistry demonstrated that C3aR and C5aR were both expressed on airway epithelial cells. Expression of the receptors was detected in the cell line A549 (Figure 4.1), in commercially bought primary human bronchial epithelial cells (HBEpC) (Figure 4.2) and in primary lung epithelial cells acquired by brushing (Figure 4.3). At least in this in vitro culture scenario, virtually 100% of epithelial cells from all sources showed evidence of both C3aR and C5aR immunoreactivity.

4.2.2 Proliferation of epithelial cells

Anaphylatoxins and cytokines were examined for their effects on the proliferation of primary HBEpC (Figure 4.4). At the concentrations employed, both IL-4 and IL-13 induced subtle but significantly elevated proliferation, while the combination was synergistic (mean of 160 % of the baseline rate; Figure 4.4A). Neither TNF-α at the concentration of 10 ng/ml employed nor 2% serum (FCS, also present in the anaphylatoxin solutions) exerted any significant effect. C3a and C5a also increased proliferation, but with the limited numbers of experiments possible this effect was statistically significant only with C3a (Figure 4.4B and 4.4C). Nanomolar concentrations of the anaphylatoxins were more effective than higher concentrations.

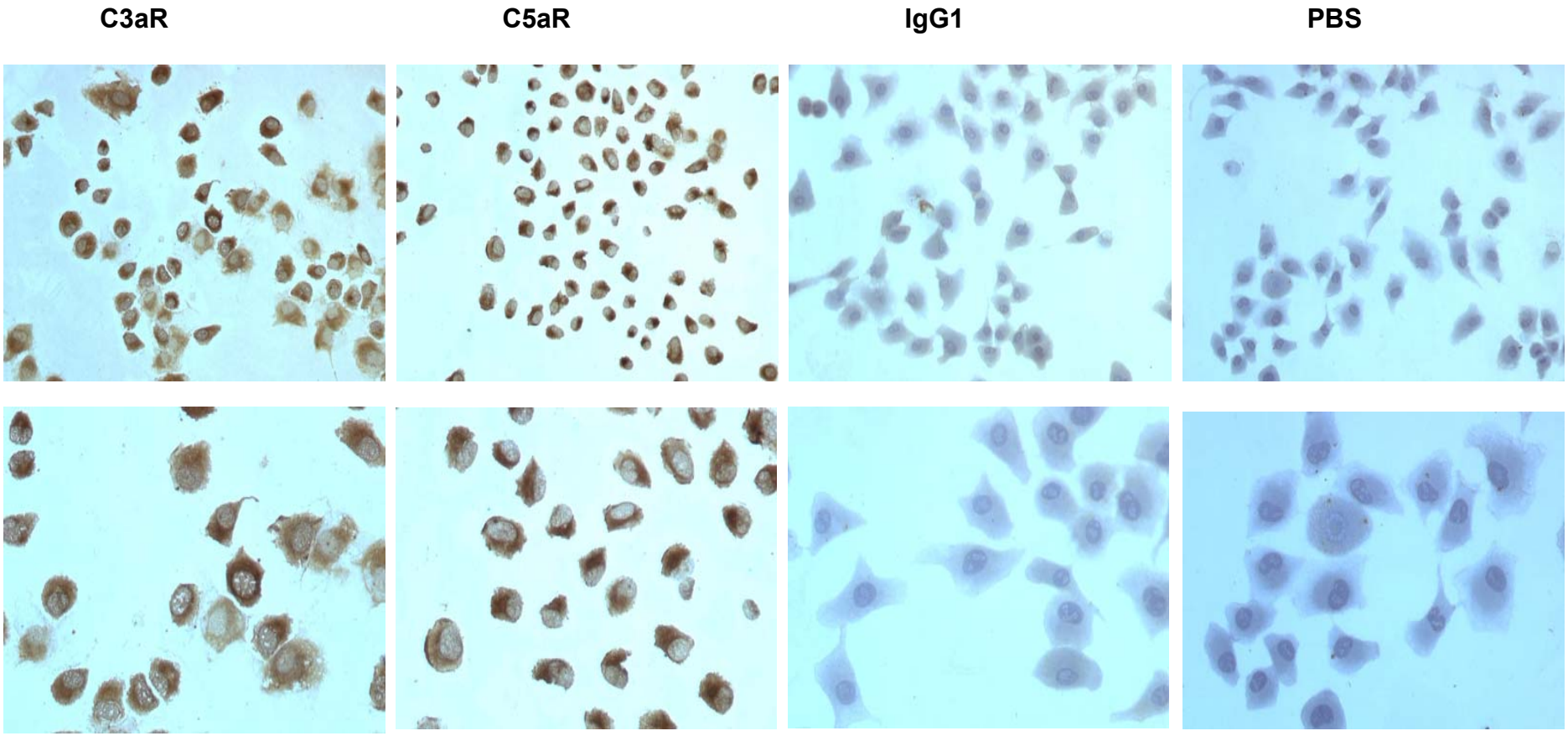

Figure 4.1 Expression of C3aR and C5aR on A549 cells by immunocytochemistry. A549 cells cultured as described in Chapter 2: Materials & Methods, section 2.1.1.1 were immunostained for the anaphylatoxin receptors C3aR and C5aR (ICC detailed in Chapter 2: Materials & Methods; section 2.2.5). Immunostaining with an isotype matched control primary antibody (IgG1) and omitting the primary antibody (PBS) were used as negative controls. The results shown are representative of 3 experiments. Magnification: top panel x10; bottom panel x20.

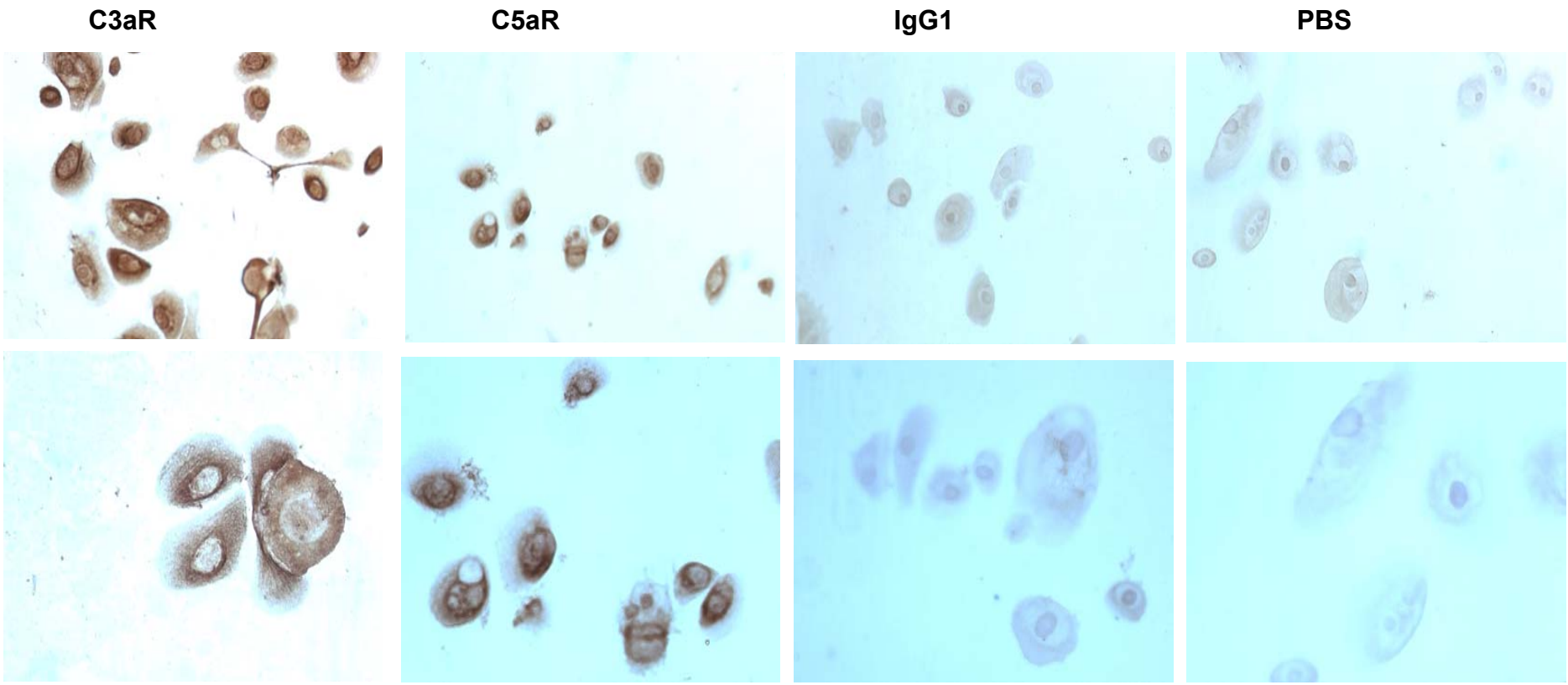

Figure 4.2 Expression of C3aR and C5aR on HBEpC by immunocytochemistry. HBEpC cultured as described in Chapter 2: Materials & Methods, section 2.1.1.3 were immunostained for the anaphylatoxin receptors C3aR and C5aR (ICC detailed in Chapter 2: Materials & Methods; section 2.2.5). Immunostaining with an isotype matched control primary antibody (IgG1) and omitting the primary antibody (PBS) were used as negative controls. The results shown are representative of 3 experiments. Magnification: top panel x10; bottom panel x20.

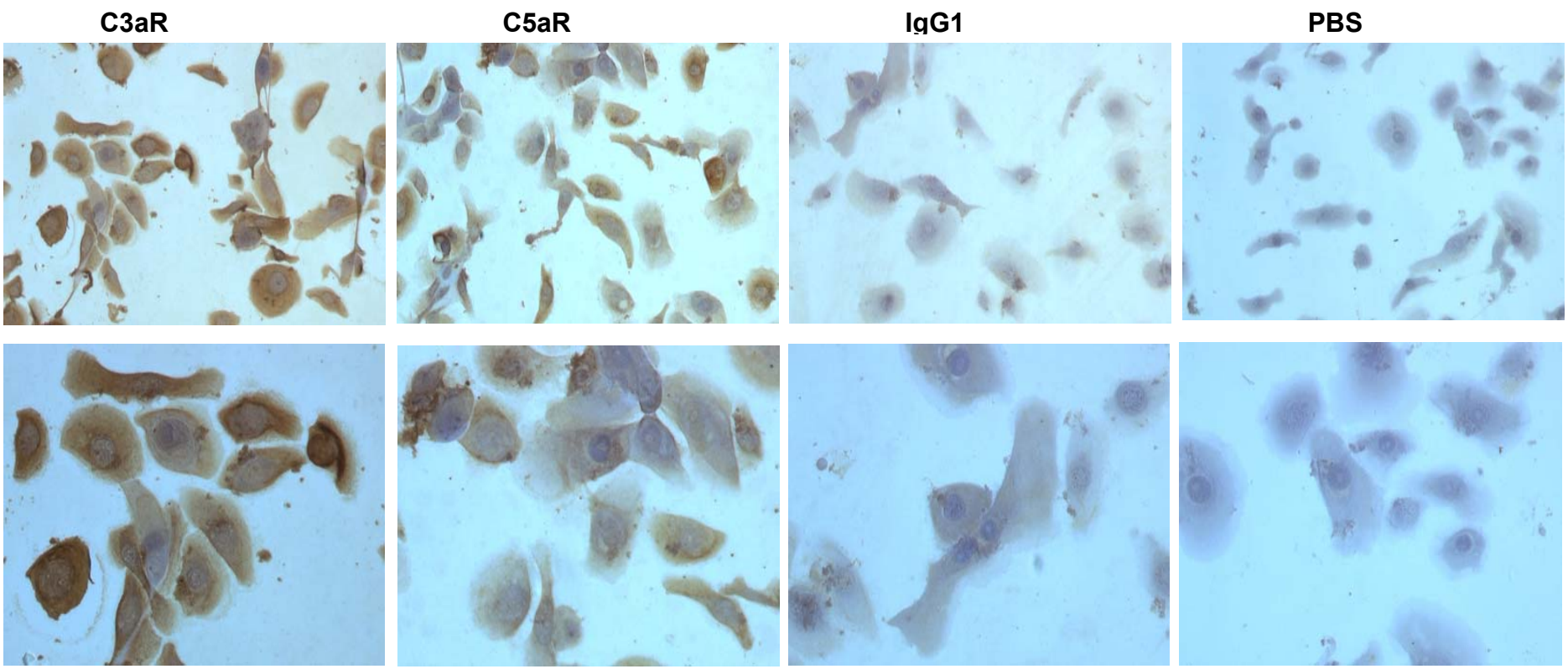

Figure 4.3 Expression of C3aR and C5aR on primary lung epithelial cells (propagated from mucosal brushings) by immunocytochemistry. Immunostaining was performed (ICC detailed in Chapter 2: Materials & Methods; section 2.2.5) for the anaphylatoxin receptors C3aR and C5aR. Immunostaining with an isotype matched control primary antibody (IgG1) and omitting the primary antibody (PBS) were used as negative controls. The results shown are representative of 3 experiments. Magnification: top panel x10; bottom panel x20.

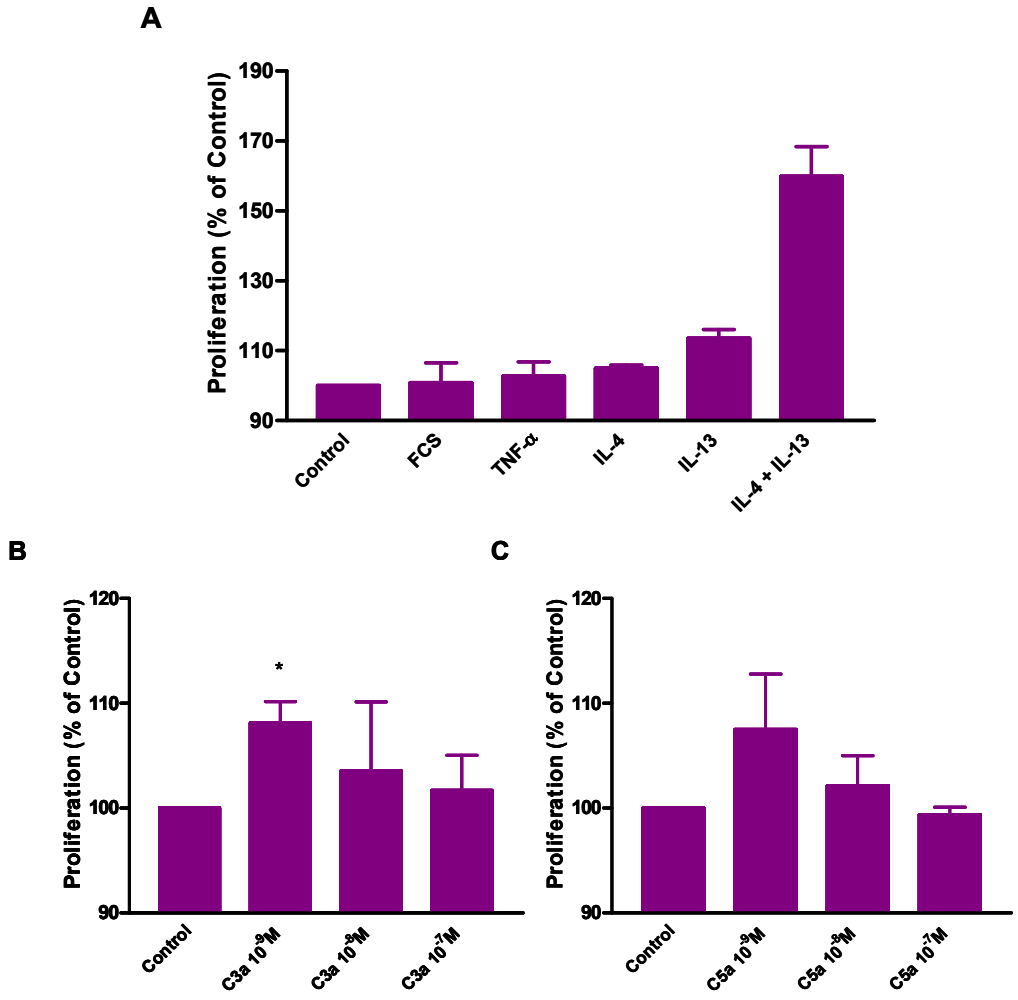

Figure 4.4 Effects of inflammatory cytokines, C3a and C5a on proliferation of HBEpC. Cells were exposed to conditions for 48 h. Conditions included treatment with A) FCS (2%) and inflammatory cytokines (at 10 ng/ml) and their combinations, and B) C3a and C) C5a at different concentrations (10^{-9}-10^{-7} M) (refer to Chapter 2: section 2.3 for details of proliferation methods). Data are expressed as mean ± SEM of the response of 3 independent MTS assay experiments, expressed as a percentage of the control cells set to 100%, * $p<0.05$ for induction of proliferation vs control (Student's t-test).

4.2.3 Complement gene expression in epithelial cells

The potential of various cytokines to induce expression of genes encoding the C3 and C5 complement components and the C3a and C5a anaphylatoxin receptors in A549 cells was measured at three time points using qPCR (Figure 4.5). The concentrations of cytokines employed were 10 ng/ml and 100 ng/ml (IFN-γ). Various cytokines both alone and in combination enhanced expression of mRNA encoding C3 (Figure 4.5A), C5 (Figure 4.5B), C3aR (Figure 4.5C) and C5aR (Figure 4.5D) by A549 cells. The time course of induction varied but in general expression of all four analytes was maximal approximately 4 hours of stimulation. In terms of expression of C3 and C3aR, IL-4 had the most prominent effect at the cytokine concentration employed. The combination of TNF-α and IL-1β was also effective. IL-4, IL-13 and IFN-γ were all effective in increasing C5 and C5aR mRNA expression. There was evidence of some synergy but the experiments were not designed or powered to detect this in fine detail.

4.2.4 FGF gene expression in epithelial cells

The potential of anaphylatoxins and Th2 cytokines to induce the expression of pro-fibrotic genes in HBEpC at the mRNA level was assessed by qPCR (Figure 4.6). Preliminary screening of FGF family genes in these cells (see discussion) suggested expression of the following FGFs and their receptors in HBEpC: FGF2, FGFR1 and FGFRL1. TNF-α stimulation of HBEpC showed late (24 h) downregulation of FGF2 expression and late (24 h) significant upregulation of FGFR1 (Figure 4.6A). IL-4 downregulated expression of FGF2 and FGFRL1 both early (4 h) and late (24 h) following stimulation (Figure 4.6B). The effects of IL-13 were similar to those of IL-4 on FGF2 expression and early FGFRL1

expression; in addition IL-13 also downregulated FGFR1 mRNA expression at 4 h (Figure 4.6C). C3a at a concentration of 10^{-8} M downregulated expression of FGF2, FGFR1 and FGFRL1 mRNA early (4 h) after exposure. By the 24 h time point expression did not significantly differ from control (Figure 4.6D). C5a at a concentration of 10^{-8} M also reduced expression of FGF2 and FGFR1 mRNA early following exposure and FGF2 and FGFRL1 mRNA later after exposure (Figure 4.6E).

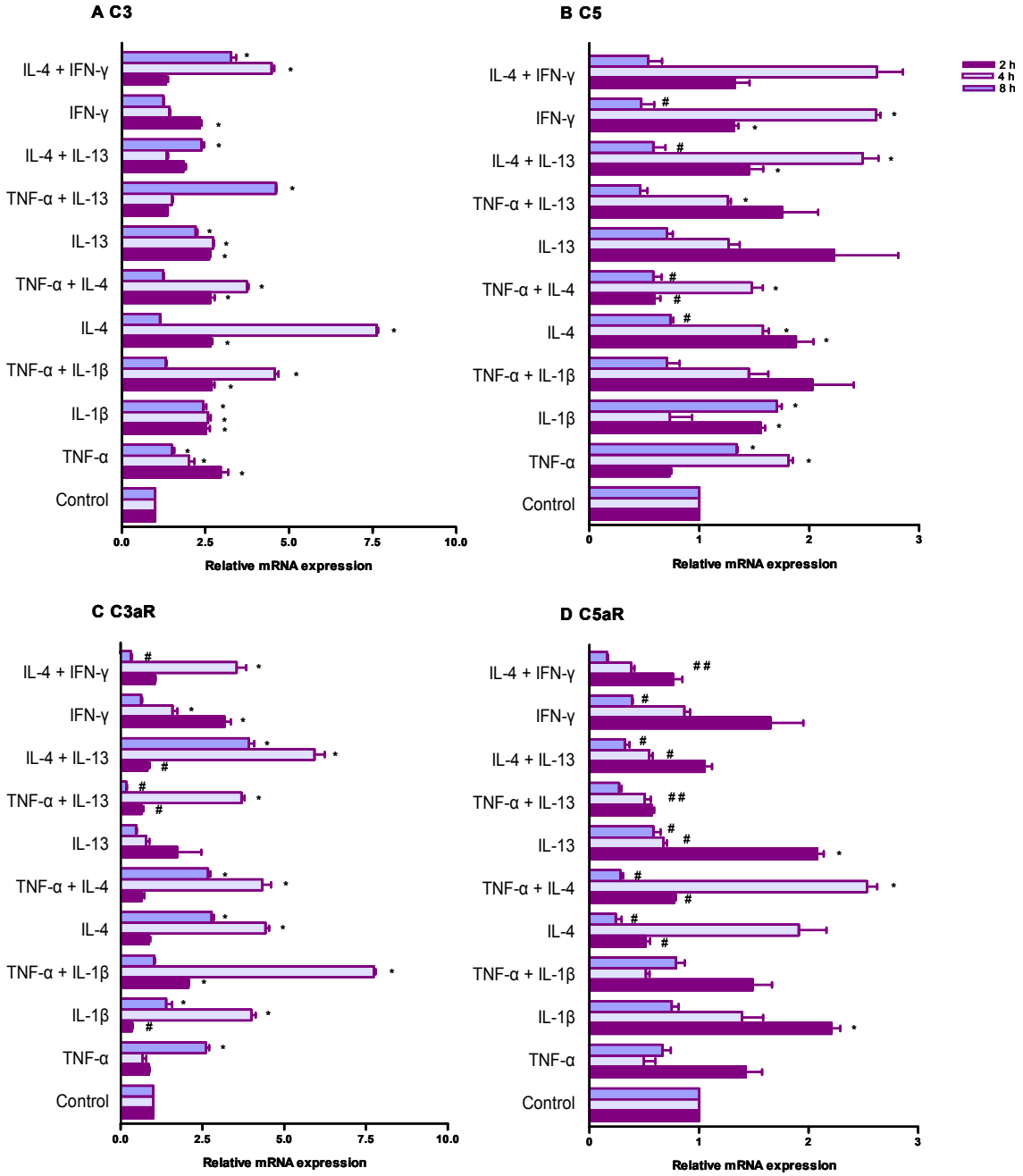

Figure 4.5 Effects of cytokines on expression of mRNA encoding complement components and receptors in A549 cells by real time qPCR. Cells were treated with a range of inflammatory cytokines (at 10 ng/ml and 100 ng/ml (IFN-γ)) for 2, 4 and 8 h to measure A) C3 B) C5 C) C3aR D) C5aR mRNA (refer to Chapter 2: section 2.4.3 for qPCR materials and methods). Data are expressed as mean ± SEM of the response of 4 independent experiments normalized relative to 18s mRNA, * $p<0.05$ increase in expression, # decrease in expression, ## decrease in all three time-points vs control (Student's t-test).

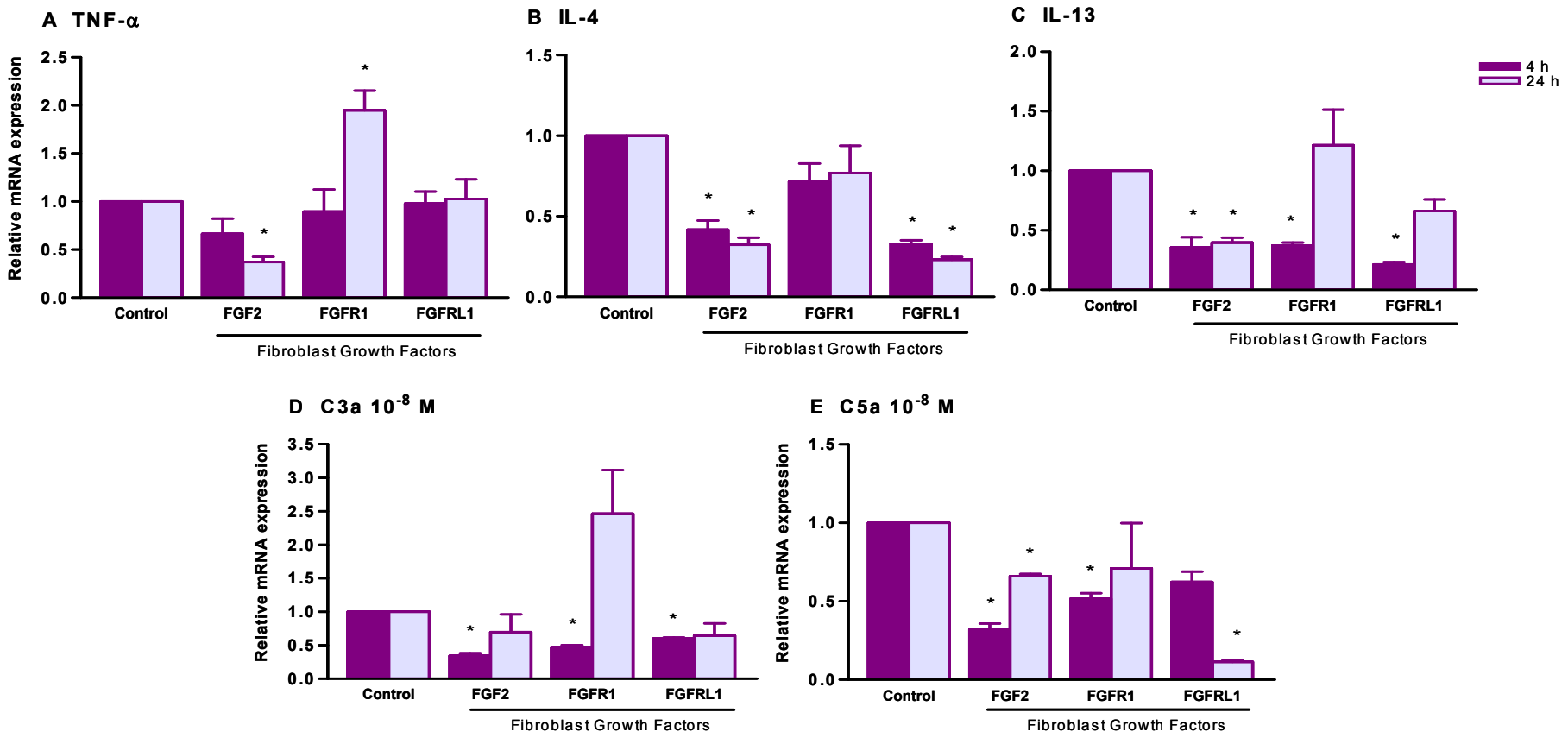

Figure 4.6 Relative expression of FGF2, FGFR1 and FGFRL1 mRNA in HBEpC by real time qPCR. Cells were treated with A) TNF-α (10 ng/ml) B) IL-4 (10 ng/ml) C) IL-13 (10 ng/ml) D) C3a 10^{-8} M E) C5a 10^{-8} M for 4 and 24 h (refer to Chapter 2: section 2.4.3 for qPCR materials and methods). Data are expressed as mean ± SEM of the response of 3 independent experiments normalized relative to 18s mRNA, * $p<0.05$ vs control cells at the same time point (Student's t-test).

4.2.5 IL-8 and IL-6 protein expression by epithelial cells

The effects of C3a and C5a on expression of IL-8 by A549 cells were examined by ELISA (Figure 4.7). These cells spontaneously produced IL-8. This production was somewhat increased by TNF-α used as a positive control but not by a range of physiological concentrations of C3a and C5a (Figure 4.7A and B); there were no statistical differences observed. Production of IL-6 and TARC by the epithelial cell lines A549 and BEAS-2B was also measured by ELISA (refer to Chapter 2: Material & Methods; section 2.5.3 for ELISA detail). No production of these proteins was detectable to the limits of the assays in the presence of a valid standard curve in the presence or absence of C3a and C5a at the same concentrations (data not shown).

4.2.6 FGF2 protein expression by epithelial cells

The effects of C3a, C5a, IL-4, IL-13 and TNF-α on the expression of FGF2 by primary HBEpC were studied using ELISA (Figure 4.8). HBEpC spontaneously produced considerable amounts of FGF2 in culture which was significantly diminished by TNF-α, IL-4, IL-13 and a range of physiological concentrations of C3a. The effect of C5a was less clear and did not show a concentration-response relationship.

4.2.7 Detection of C3a and C5a in epithelial cells

The effects of proinflammatory and Th2 cytokines (TNF-α, IL-4 and IL-13 at 10 ng/ml) on the expression of C3a and C5a in A549 cells was examined by Western blotting (see Chapter 2: Methods section 2.5.4 for Western blotting

details). The cytokines did not induce detectable expression of either anaphylatoxin.

4.2.8 Detection of FGFR1 in epithelial cells

The effects of C3a, C5a and the Th2 cytokines (IL-4 and IL-13 at 10 ng/ml) on the expression of FGFR1 in HBEpC was examined by Western blotting (see Chapter 2: Methods section 2.5.4 for details). Neither the anaphylatoxins nor the cytokines were found to induce detectable expression of this growth factor receptor under the conditions employed (data not shown).

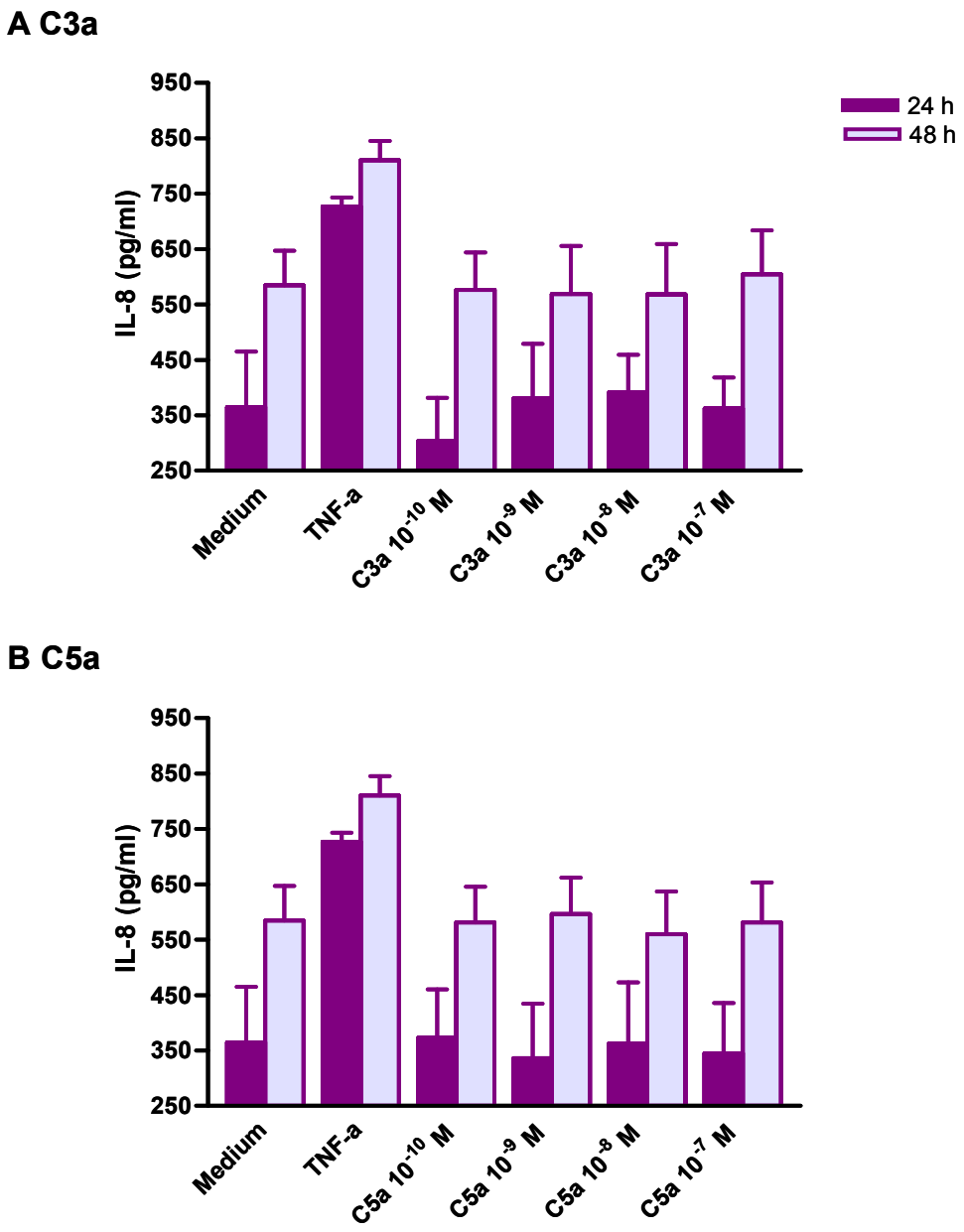

Figure 4.7 Effects of C3a and C5a on IL-8 production in A549 cells. A549 cells were stimulated for 24 and 48 h with various concentrations of A) C3a and B) C5a ranging from 10^{-10} M to 10^{-7} M, and TNF-α at 10 ng/ml as a positive control. Supernatants were harvested and IL-8 expression was measured by ELISA (refer to Chapter 2: section 2.5.3 for details of ELISA method). Data are expressed as the mean ± SEM of 3 independent experiments. Statistical significance was assessed by Student's t-test; there were no statistical differences.

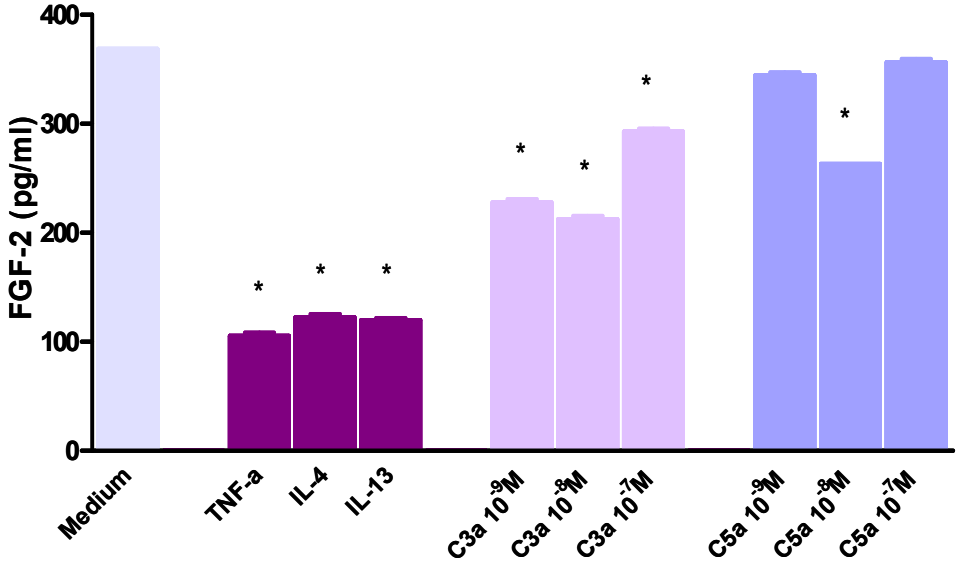

Figure 4.8 Effects of inflammatory cytokines, C3a and C5a on FGF2 expression by HBEpC. Cells were cultured and stimulated for 48 h with various concentrations of C3a and C5a ranging from 10^{-9} M to 10^{-7} M and IL-4 and TNF-α at 10 ng/ml. Supernatants were harvested and FGF2 expression was assessed by ELISA as described in Chapter 2: Materials & Methods, section 2.5.3. Data are expressed as mean ± SEM of 3 independent experiments, * $p<0.05$ vs medium control (Student's t-test).

4.3 Discussion

The position and location of the airway epithelium makes it an important cell to be studied in asthma. The bronchial epithelium is in a key position to translate and coordinate inflammatory signals between the luminal space and the lung tissue (Hahn *et al.* 2006). Epithelial cells play important roles in host defence, inflammation, and regulation of immune responses (Schleimer *et al.* 2007).

Epithelial cells act as an initiator, mediator, and regulator in innate and adaptive immune responses, as well as the transition from innate immunity to adaptive immunity (Wang *et al.* 2008). Although asthma is an inflammatory disorder of the conducting airways involving Th2-type T cells, the epithelium also plays an important role in orchestrating the inflammatory response by interacting with multiple environmental factors to produce a chronic wound scenario involving tissue injury and aberrant repair (Wang *et al.* 2008). Part of this is a primary disruption of epithelial tight junctions that allows inhaled substances to pass more easily into the airway wall to interact with immune and inflammatory cells. Aberrant communication between the damaged and stressed epithelia leads to the generation of growth factors that interact with the underlying mesenchyme to promote airway remodelling responses and a more chronic and persistent inflammatory phenotype (Wang *et al.* 2008). Disordered epithelial function with reduced antioxidant defence and impaired capacity to produce primary IFN-γ may also account for asthmatic susceptibility to air pollution and respiratory virus infection, respectively (Holgate 2007).

In this chapter, it has been demonstrated that cell lines such as A549, primary lung epithelial cells and mucosal cells outgrown in our own laboratory express receptors for both anaphylatoxins C3aR and C5aR *in vivo* and *in vitro*. This is

not a completely new finding; existing studies have used other means to demonstrate expression of the receptors and anaphylatoxins such as in situ hybridisation (Drouin *et al.* 2001b) and *in vivo* studies showing C3a and C5a upregulation in the BAL fluid in asthmatic patients (Krug *et al.* 2001). Drouin and colleagues reported positive C3aR and C5aR immunohistochemistry in the bronchioles of normal human lung. The signal was localized to the apical surface of the bronchial epithelial cells. They also demonstrated positive immunohistochemical staining for C3aR and C5aR in cultured human bronchial epithelial cells. Furthermore, they established that both receptors are up-regulated in two distinct murine models of lung inflammation: endotoxaemia and OVA-induced asthma (Drouin *et al.* 2001b). Despite these studies there have in contrast been few if any studies of the functional implications of this expression.

In like manner to Th2 cytokines such as IL-4 and IL-13, C3a and C5a increased epithelial cell proliferation, although the effect was clearest with C3a. Disordered epithelial proliferation is thought to be a fundamental feature of asthma pathogenesis, leading to the concept of the EMT unit: the airway epithelium is in a key position to regulate the activity of the underlying cell layers and their response to external stimuli (Holgate *et al.* 2000). The importance of epithelial–mesenchymal interaction is emphasized in the pathophysiological concepts of asthma (Holgate *et al.* 2000), idiopathic pulmonary fibrosis, and other extra-pulmonary diseases (Hostettler *et al.* 2008). Epithelial hyperplasia may be partly responsible for the progressive decline in lung function which can be observed in some chronic asthmatics; although other processes such as distal airway remodelling and air trapping may also contribute (Cohen *et al.* 2007). It should be noted, however, that as with many “remodelling” changes in asthma there is very little direct evidence that they impact on lung function. Benayoun and coworkers previously reported in a small group of subjects with severe asthma

that there was a difference in subepithelial basement membrane thickness and epithelial integrity in comparison to normal subjects and subjects with mild–moderate asthma (Benayoun *et al.* 2003). Vignola and colleagues demonstrated in oral corticosteroid-dependent subjects with asthma that there was increased epithelial thickness in comparison to subjects with untreated asthma, although there was no difference in comparison to normal control subjects (Vignola *et al.* 2001). Pepe and associates also demonstrated a non-significant trend to greater subepithelial fibrosis in subjects with severe asthma compared with those with moderate asthma but found no evidence of epithelial hyperplasia (Pepe *et al.* 2005).

The propensity of C3a to induce epithelial proliferation observed in the present study may be particularly relevant to the effects of allergen exposure, since Ricciardolo and colleagues demonstrated that allergen challenge appears to increase epithelial proliferation in subjects with mild asthma (Ricciardolo *et al.* 2003).

Multiple cytokines, particularly IL-4, TNF-α and IL-1β increased C3 and C3aR mRNA expression in epithelial cells, while IL-4, IL-13 and IFN-γ increased C5 and C5aR mRNA, underlining the possibility that epithelial cells, particularly in an inflammatory environment such as asthma, are potentially capable of producing complement components de novo as well as responding to them. This extends previous observations, previously only made in immortalised epithelial cell lines that various asthma-relevant and other cytokines including IL-4, IL-13, TNF-α and IFN-γ can induce C3 production in A549 and BEAS-2B cells (Khirwadkar *et al.* 1993;Zhao *et al.* 2000;Varsano *et al.* 2000;Christian-Ritter *et al.* 1994). Khirwadkar and colleagues showed that A549 cells produce multiple complement components in response to cytokine stimulation; for example IL-4

enhanced C3 production (Khirwadkar *et al.* 1993). Zhao et al have also shown that the proinflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-γ act as potent inducers of C3 in A549 cells (Zhao *et al.* 2000). Similarly, Varsano and colleagues showed that BEAS-2B cells spontaneously generate C3, but not C5 in serum-free media, and that this was enhanced by TNF-α stimulation (Varsano *et al.* 2000). Christian-Ritter and colleagues reported that IL-4 increases C3 synthesis by A549 cells in a time- and concentration- dependent fashion, and maximal increase in C3 synthesis occurred after stimulation of A549 cells with IL-4 at 10 ng/ml for 3 days, justifying the use of this cytokine at this concentration in the present study (Christian-Ritter *et al.* 1994). As in the present study, the relative abundance of C3 mRNA in A549 cells was found to increase following IL-4 treatment, indicating that its effects on C3 production are pretranslational (Christian-Ritter *et al.* 1994). The new findings in the present study suggest that asthma-relevant cytokines may increase the production of C3 and C5 by primary bronchial epithelial cells as well as their ability to respond to derived fragments of these products including C3a and C5a. Thus some of the remodelling activities of IL-4 and other cytokines on epithelial cells could conceivably be mediated partly through the effects of complement.

Similarly to the remodelling cytokines IL-4 and IL-13, C3a and C5a tended to reduce production of mRNA encoding FGF2, FGFR1 and FGFRL1 by primary bronchial epithelial cells. These particular growth factors and their receptors were singled out for study because preliminary screening of all the FGFs and their receptors at the mRNA level showed that only FGF2, FGFR1 and FGFRL1 mRNA were detectable in the epithelial cells before or after stimulation. Both FGFR1 and FGFRL1 (the most recently discovered member of the FGF receptor family, also known as FGFR5) are capable of binding all FGF ligands, but FGFRL1 differs from FGFR1 in not possessing a cytoplasmic split tyrosine

kinase domain. Both function to control the proliferation, differentiation and migration of structural cells in various tissues. The situation is further complicated by the fact that some FGFs, including FGF2 can activate receptors both intracellularly and extracellularly. The concentration of cytokines used as positive controls (generally 10 ng/ml), but 100 ng/ml in the case of IFN-γ were chosen because most of the existing literature shows that these particular concentrations are optimal for induction of growth factors.

Growth factors such as FGF2 are secreted by epithelial cells. HBEpC spontaneously produced high amounts of FGF2 in culture. It is of great interest that complement components especially C3a appear to have the capacity to reduce the production by epithelial cells of this key growth factor implicated in airways remodelling. The airway epithelium is considered a principal source of FGF2 in the airways (Shute *et al.* 2004). Its elevated expression in asthmatic airways has been confirmed by immunohistochemistry in humans (Shute *et al.* 2004) and in a nonhuman primate model (Bosse *et al.* 2006) and inflammatory leukocytes including mast cells, macrophages and eosinophils have also been reported to produce it (Bosse *et al.* 2006). Thus complement components could in theory inhibit local epithelial production of FGF2 in the asthmatic airway at least in some situations.

In contrast to growth and remodelling factors, C3a and C5a did not affect production of the chemokine IL-8 by bronchial epithelial cells, while IL-6 production could not be detected under any of the conditions employed. This contrasts with a previous report (Kashyap *et al.* 2002) which provided precedent for the possibility that such effects may be observed and the suggestion that C5a stimulates IL-8 production by human bronchial epithelial cells (Kashyap *et al.* 2002). In addition, bronchial epithelial cells have been reported to over

produce IL-6 in asthmatics (Marini *et al.* 1992). Production of the chemokine TARC was also examined based on previous reports that epithelial cells are a source of this chemokine (Laberge & El Bassam 2004) and that it is upregulated in the BEAS-2B airway epithelial cell line by cytokines including IL-4, IL-13 and TNF-α and also in vivo in the asthmatic bronchial mucosa (Sekiya *et al.* 2000) where the expression of TARC immunoreactivity is increased of subjects with asthma as compared to normal controls and its expression is prominent in epithelial cells (Sekiya *et al.* 2000). In addition IL-4 and IL-13 alone or in combination with TNF-α have been reported to induce the production of eotaxin and MCP-4 by bronchial epithelial cells (Matsukura *et al.* 2001). All of these chemokines have been reported to be over expressed in asthmatic airways compared with controls *in vivo* (Sekiya *et al.* 2000). In addition to being chemoattractants for leukocytes, the chemokine TARC may recruit CCR4+ Th2 T cells which in turn are induced to produce more Th2 cytokines, establishing a mechanism for amplifying Th2 responses (Laberge & El Bassam 2004). In our hands C5a and C3a at a range of concentrations did not augment IL-8 release from A549 cells, while IL-6 and TARC release from A549 and BEAS-2B cells could not be detected. These discrepancies might result from the fact that these cell lines were immortalised and may have lost functional genes, for example those encoding anaphylatoxin receptors or particular cytokines. It is unfortunate that primary cell lines were not further examined in this regard. This was because this material is much more scarce and preliminary screens with HBEpC also failed to detect IL-8 protein production.

In summary, evidence has been presented consistent with the hypothesis that anaphylatoxin complement components contribute to airway remodelling by altering epithelial cellular proliferation and their production of key remodelling mediators. This is widely regarded as a fundamental mechanism of asthma

pathogenesis, where epithelial remodelling appears to be inherently abnormal. Functionally, while complement components could in theory augment epithelial proliferation for example following allergen exposure, it appears that they may also reduce the production of key pro-fibrotic mediators such as FGF2.

Chapter 5: Fibroblast Cells

5.1 Introduction

Fibroblasts are the major type of mesenchymal cell present in the matrix of connective tissue. Fibroblasts provide structure and function to maintain integrity, form and solidarity of lung tissue (Spoelstra, Postma, & Kauffman 2001). Fibroblasts likely bring about some of the changes associated with inflammation and remodelling in asthma and may also recruit and activate inflammatory cells.

Airway inflammation develops in airway tissues in response to exposure to potentially injurious physical or biological agents. This response includes the release of various mediators, among which cytokines and chemokines are considered to play major regulatory roles. Fibroblasts, in addition to other tissue structural cells, may actively participate in this process by releasing a variety of cytokines and chemokines (Takahashi *et al.* 2006). Fibroblasts are capable of responding to a range of mediators, and producing a vast variety of chemokines and cytokines upon stimulation, such as IL-8 and TNF-α, which in turn, are able to activate inflammatory cells to the lung (Laberge & El Bassam 2004).

Activation of fibroblasts by various mediators alters their phenotype towards a transitional mesenchymal fibroblast/smooth muscle phenotype termed a myofibroblast. Activation of fibroblasts leads to a shift in fibroblast phenotype (Spoelstra, Postma, & Kauffman 2001). Airway remodelling is as a result of a change in the fibroblasts in the lung.

Fibroblasts from chronically inflamed tissue differ from fibroblasts obtained from normal tissues in cytokine production, pattern of gene expression of matrix proteins as well as the rate of cell proliferation (Benayoun *et al.* 2003).

Increased markers of fibroblasts have been reported on the bronchial mucosa of asthmatics: accumulation seems to be proportional to disease severity (Benayoun *et al.* 2003). This is thought to be a key feature of remodelling. Although in any healing response fibroblasts are attracted to subepithelial compartments of mucosal surfaces to effect tissue repair and healing, in asthma this appears to become uncontrolled with increased lay down of matrix proteins, a phenomenon which is postulated ultimately to result in diminution of lung function with time (Crosby & Waters 2010) (Ingram *et al.* 2011).

Subepithelial fibrosis is a prominent feature of airway remodelling the severity of which has been linked, at least in cross-sectional studies, with asthma severity (Saito *et al.* 2003). Subepithelial fibrosis appears to be affected by myofibroblasts, which in addition to adding to fibrous protein lay down also have contractile properties of their own, thus contributing to the airway narrowing and airflow limitation in asthma (Saito *et al.* 2003).

It is possible that anaphylatoxins are one of the stimuli to drive fibroblast proliferation and differentiation in asthma, with consequent effect on mediators, such as IL-8 and FGF2 which induce tissue remodelling.

The specific aims of the studies described in this chapter were to:

1. Assess the expression of C3aR and C5aR on human lung fibroblasts;
2. Assess the proliferative response of fibroblast cells to stimulation with anaphylatoxins and Th2 cytokines;

3. Examine C3a and C5a-stimulated production of important remodelling mediators (growth factors) by fibroblasts at the mRNA and protein level, comparing with Th2-type cytokine-stimulated production as a positive control.

5.2 Results

5.2.1 Expression of complement receptors on lung fibroblasts

Immunocytochemistry demonstrated expression of C3aR and C5aR immunoreactivity on human pulmonary fibroblast cells (HPFC) (Figure 5.1) cultured and stained as described in Chapter 2: Materials & Methods, section 2.1.2.2.

5.2.2 Proliferation of lung fibroblasts

Anaphylatoxins and Th2 cytokines were examined for their effects on the proliferation of human pulmonary fibroblast cells (Figure 5.2). Of the cytokines, TNF-α, IL-4 and IL-13 significantly increased fibroblast proliferation, although IL-4 was most effective at the particular concentration of 10 ng/ml employed, increasing proliferation to a mean of 208.09 ± 7.16 % of baseline (Figure 5.2A). IL-4 and IL-13 in combination also induced proliferation although this was similar to TNF-α and surprisingly not additive or synergistic. C3a significantly increased fibroblast proliferation in the region of 113 % of baseline at a range of concentrations embracing the physiological, although in general this effect was not as impressive as that of the cytokines and the concentration/response relationship was poor owing to inherent variability of the data (Figure 5.2B). C5a at the highest concentration employed (10^{-7} M) induced increased fibroblast proliferation (Figure 5.2C).

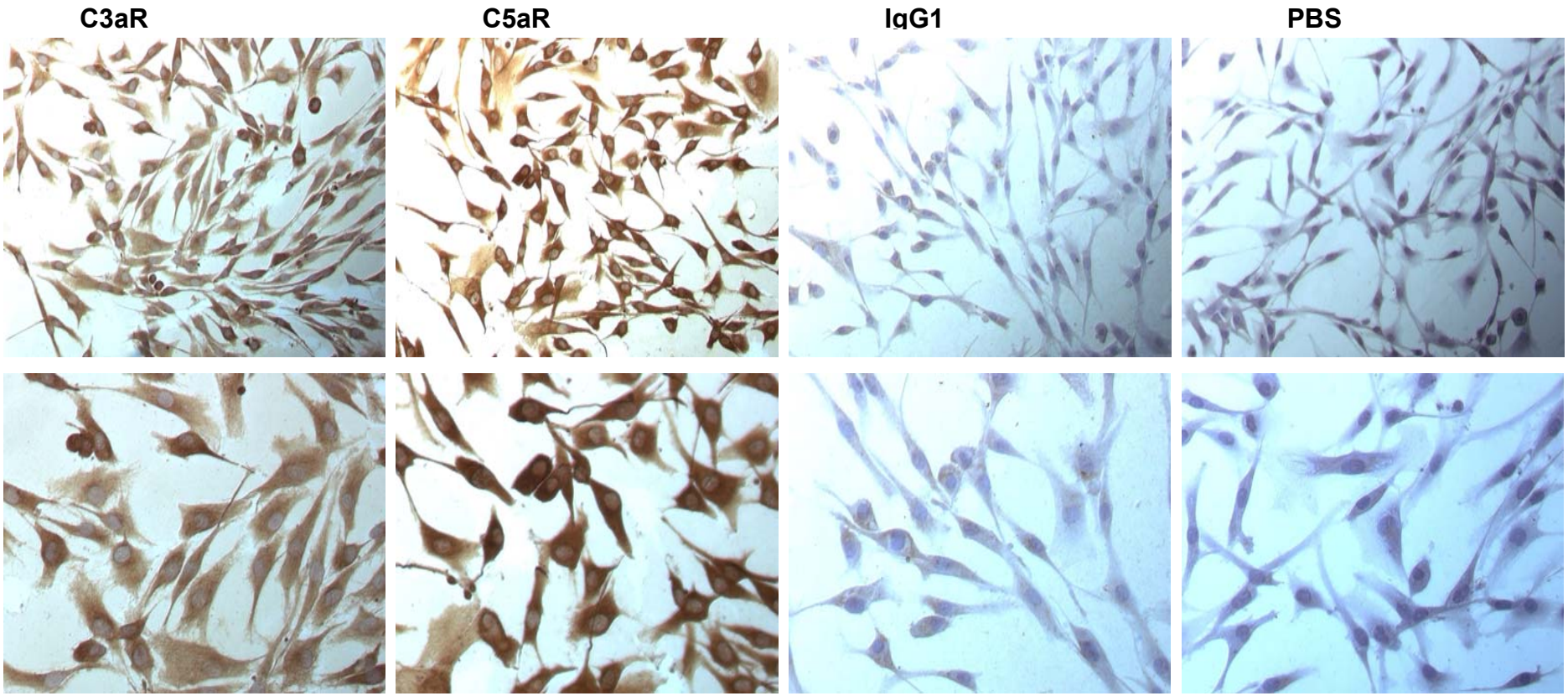

Figure 5.1 Expression of C3aR and C5aR on HPFC by immunocytochemistry. Lung fibroblasts cultured as described in Chapter 2: Materials & Methods, section 2.1.2.2 were immunostained for the anaphylatoxin receptors C3aR and C5aR (ICC detailed in Chapter 2: Materials & Methods, section 2.2.5). Immunostaining with an isotype matched control primary antibody (IgG1) and omitting the primary antibody (PBS) were used as negative controls. The results shown are representative of 4 experiments. Magnification: top panel x10; bottom panel x20.

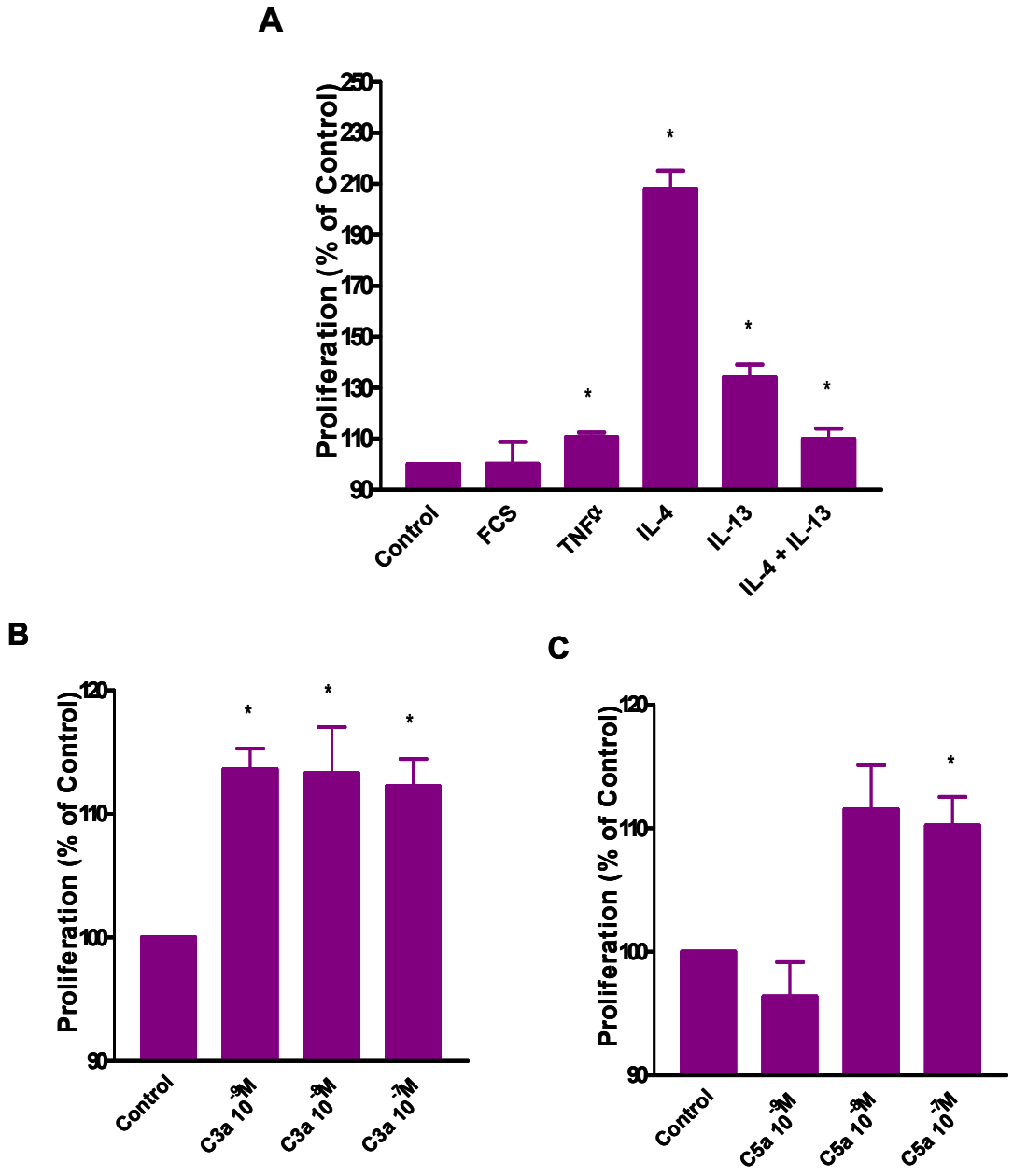

Figure 5.2 Effects of cytokines, C3a and C5a on proliferation of HPFC. Cells were exposed to conditions for 48 h. Conditions include treatment with A) FCS (2%) and inflammatory cytokines (at 10 ng/ml) and B) C3a and C) C5a at different concentrations (10^{-9}-10^{-7} M) (refer to Chapter 2: Materials & Methods, section 2.3 for detail on proliferation method). Data are expressed as mean ± SEM of the response of 3 independent MTS assay experiments, expressed as a percentage of the control cells set to 100%, * $p<0.05$ for induction of proliferation vs control (Student's t-test).

5.2.3 FGF gene expression in lung fibroblasts

The ability of anaphylatoxins and cytokines to induce expression of FGFs and their receptors in primary human lung fibroblasts was measured 4 and 24 hour following stimulation by qPCR (Figure 5.3). The particular products analysed were defined from a more preliminary screen (see discussion). At the concentrations of cytokines employed, TNF-α had little consistent effect on the expression of mRNA encoding FGF2, FGF11, FGFR1 and FGFRL1 (Figure 5.3A). Both IL-4 and IL-13 suppressed fibroblast production of mRNA encoding all of these analytes early (4 h) after stimulation, although IL-13 was particularly effective in reversing this after 24 hours (Figure 5.3C). C3a at a concentration of 10^{-8} M had no significant effect on the expression of mRNA encoding the FGFs, while C5a at the same concentration increased expression of mRNA encoding FGF11 and FGFR1 and decreased expression of FGF2 (Figure 5.3D and 5.3E).

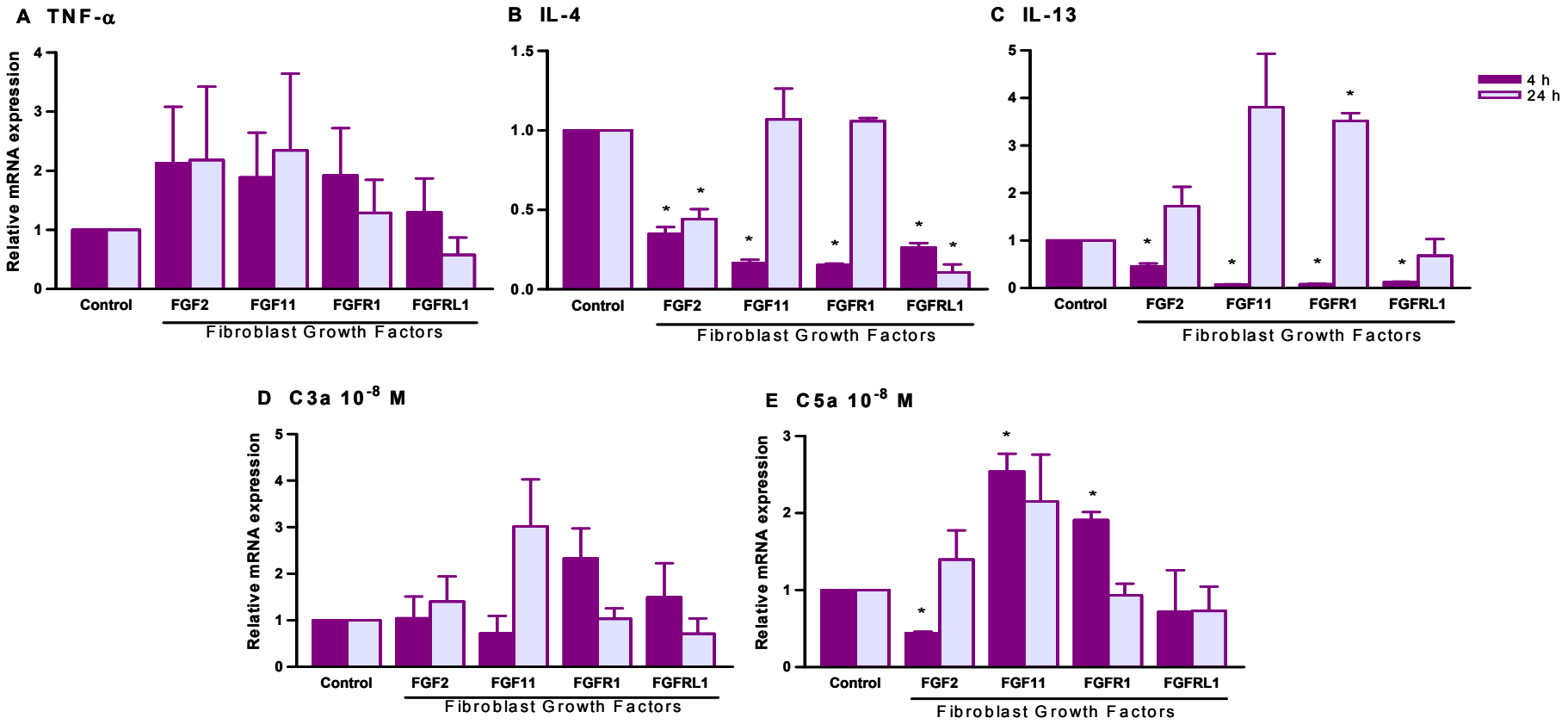

Figure 5.3 Relative expressions of FGF2, FGF11, FGFR1 and FGFRL1 mRNA in HPFC by real time qPCR. Cells were treated with A) TNF-α (10 ng/ml) B) IL-4 (10 ng/ml) C) IL-13 (10 ng/ml) D) C3a 10^{-8} M E) C5a 10^{-8} M for 4 and 24 h (refer to Chapter 2: section 2.4.3 for qPCR materials and methods). Data are expressed as mean ± SEM of the response of 3 independent experiments normalized relative to 18s mRNA, * $p<0.05$ compared with control cells at the same time point (Student's t-test).

5.2.4 IL-6 and IL-8 protein expression by fibroblast cells

The effects of C3a and C5a on expression and release of IL-6 (Figure 5.4) and IL-8 (Figure 5.5) were examined in the human fibroblast cell line MRC-5 by ELISA. Robust production of IL-6 and IL-8 (above 700 pg/ml) was observed in response to TNF-α used as a positive control. In contrast, neither C3a nor C5a at a range of concentrations embracing the physiological significantly altered the production of IL-6 or IL-8 by these cells with the exception of C3a at the highest concentration tested (10^{-7} M) which significantly increased IL-6 production. Inexplicably, C3a also increased IL-8 production at 10^{-9} M but there was no concentration/response effect.

5.2.5 FGF2 protein expression by fibroblast cells

The effects of C3a, C5a and cytokines on the expression and release of FGF2 was studied in HPFC by ELISA (Figure 5.6). Fibroblasts spontaneously released FGF2. Production was increased under the conditions employed by IL-4 and decreased by IL-13, but TNF-α had no significant effect. Both C3a and C5a at a range of concentrations embracing the physiological exerted opposing effects on FGF2 production by HPFC: C3a and C5a at the higher concentrations employed increased and decreased FGF2 protein expression respectively, whilst C3a and C5a at the lower concentrations employed decreased and increased FGF2 protein expression respectively.

5.2.6 Expression of FGFR1 by fibroblast cells

The effects of C3a, C5a and cytokines on the expression of FGFR1 by HPFC was investigated by Western blotting (Figure 5.7). FGFR1 (92 kDa) was

detected in the treated HPFCs showing that HPFC do express FGFR1. However a concentration/response effect for the anaphylatoxins was not observed following densitometric evaluation, nor did the cytokines have an effect hence the exclusion of numerical data. For the western blot three replicates were done.

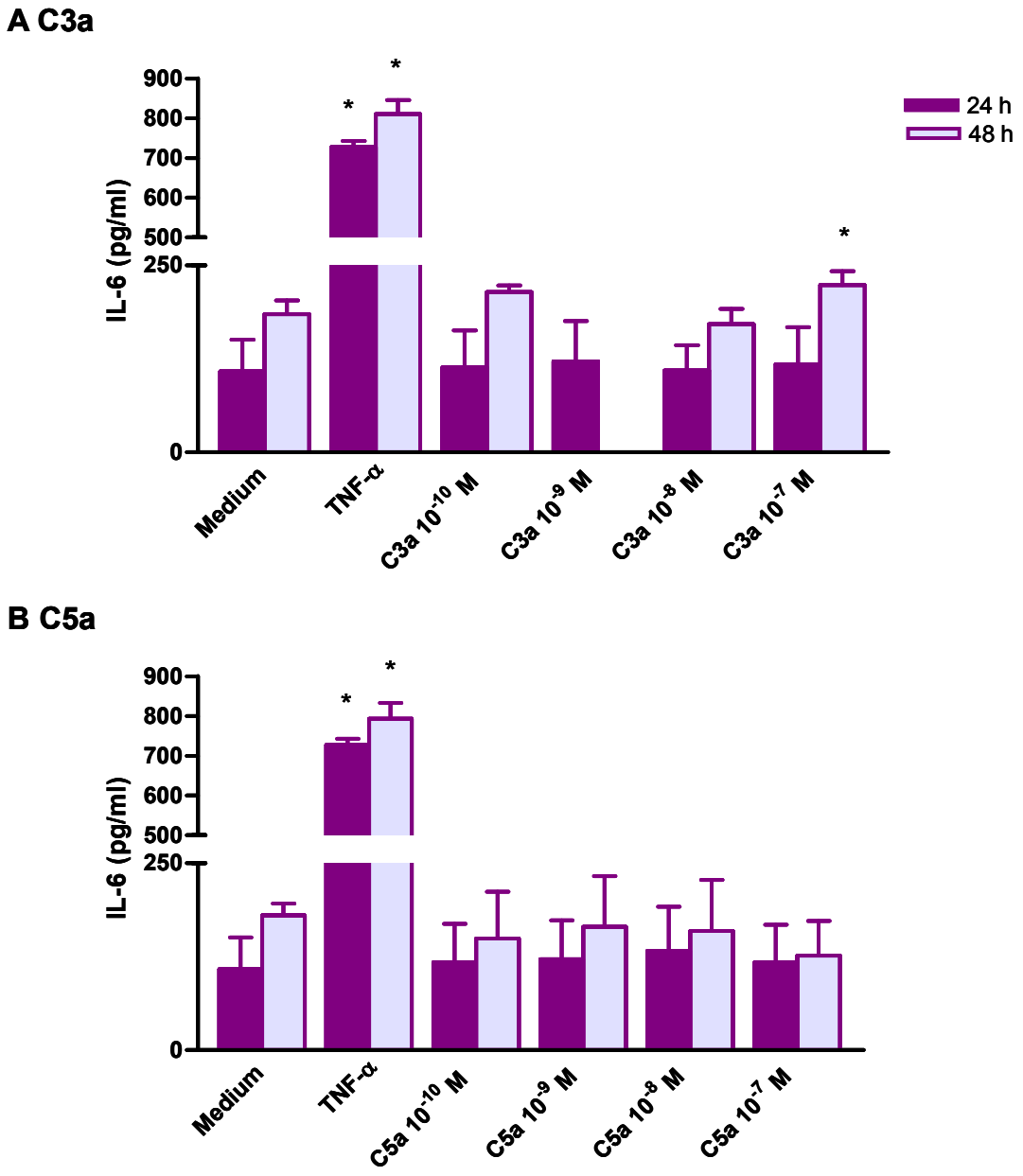

Figure 5.4 Effects of C3a and C5a on IL-6 production in Fibroblasts. MRC-5 cells were cultured as described in Chapter 2: Materials & Methods, section 2.1.2.1 and stimulated for 24 and 48 h with various concentrations of A) C3a and B) C5a ranging from 10^{-10} M to 10^{-7} M, and TNF-α at 10 ng/ml as a positive control. Supernatants were harvested and IL-6 expression was measured by ELISA (refer to Chapter 2: section 2.5.3 for details of ELISA method). Data are expressed as mean ± SEM of the response of 3 independent experiments, * $p<0.05$ compared with medium control at the same time point (Student's t-test).

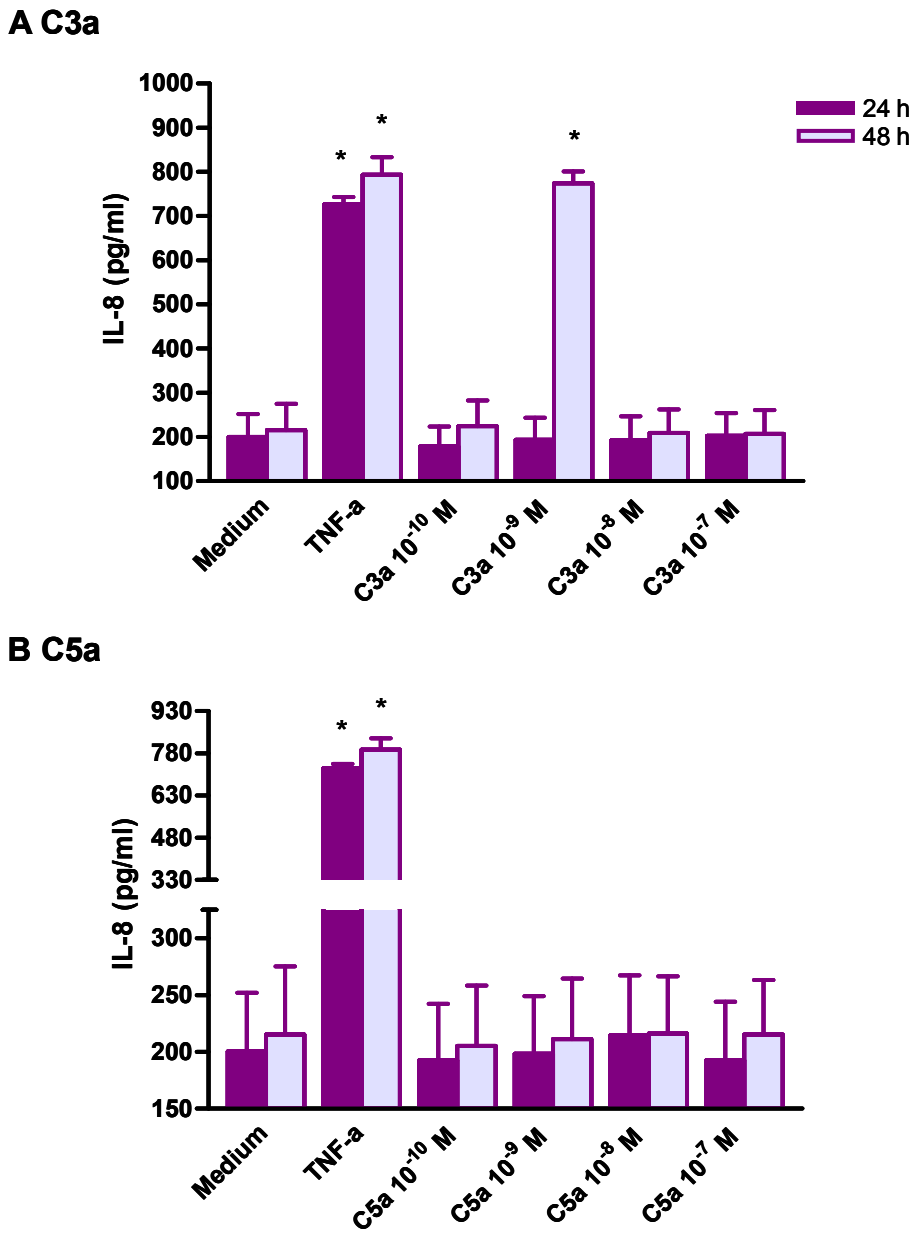

Figure 5.5 Effects of C3a and C5a on IL-8 production in Fibroblasts. MRC-5 cells were cultured as described in Chapter 2: Materials & Methods, section 2.1.2.1 and stimulated for 24 and 48 h with various concentrations of A) C3a and B) C5a ranging from 10^{-10} M to 10^{-7} M, and TNF-α at 10 ng/ml as a positive control. Supernatants were harvested and IL-6 expression was measured by ELISA (refer to Chapter 2: section 2.5.3 for details of ELISA method). Data are expressed as mean ± SEM of the response of 3 independent experiments, * $p<0.05$ compared with medium control at the same time point (Student's t-test).

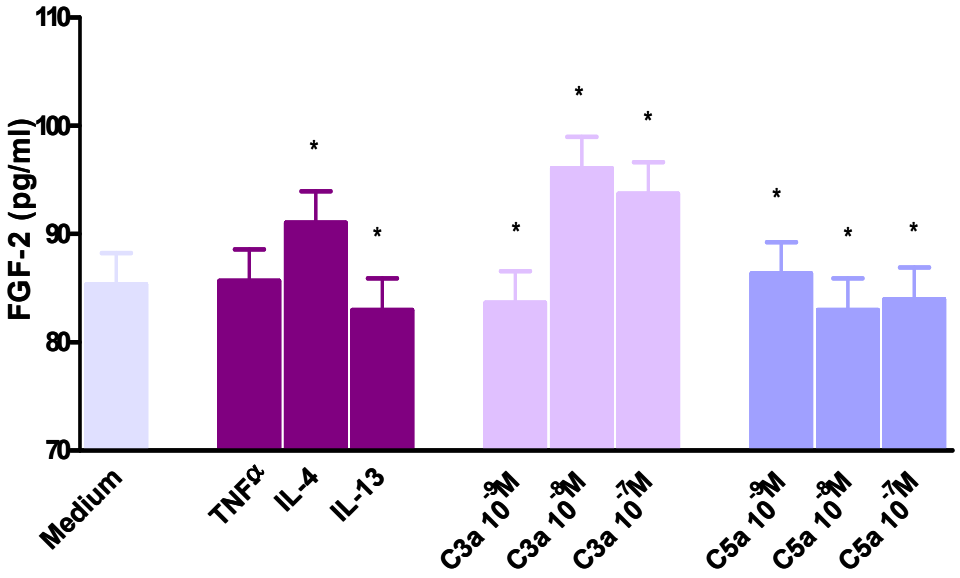

Figure 5.6 Effects of inflammatory cytokines, C3a and C5a on FGF2 expression by HPFC. Cells were cultured and stimulated for 48 h with various concentrations of C3a and C5a ranging from 10^{-9} M to 10^{-7} M and Th2 cytokines and TNF-α at 10 ng/ml. Supernatants were harvested and FGF2 expression was assessed by ELISA as described in Chapter 2: Materials & Methods, section 2.5.3. Data are expressed as mean ± SEM of the response of 3 independent experiments, * $p<0.05$ compared with medium control (Student's t-test).

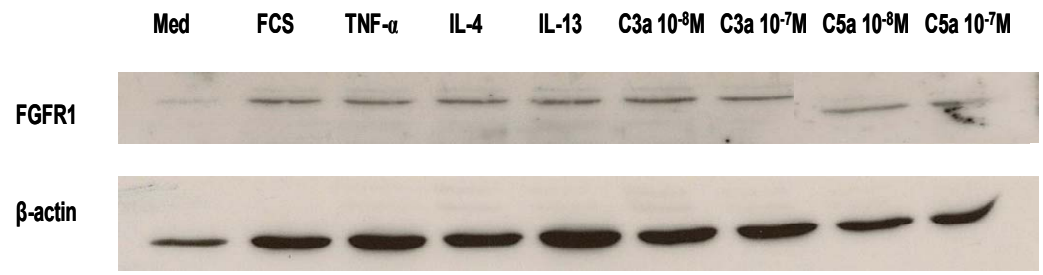

Figure 5.7 Effects of inflammatory cytokines, C3a and C5a on FGFR1 expression on HPFC as assessed by Western blot. Cells were stimulated with TNF-α and Th2 cytokines at 10 ng/ml, and C3a and C5a at two different concentrations (Western blotting method detailed in Chapter 2: section 2.5.4). Western blot (of 24 h stimulation) is representative of 3 independent experiments; β-actin was used as control.

5.3 Discussion

The data in this chapter clearly demonstrate for the first time that human pulmonary fibroblasts express receptors for both anaphylatoxins C3a and C5a. In addition, along with IL-4 and IL-13 which are well established “remodelling” cytokines, C3a and to a more tentative extent C5a were capable of increasing the proliferation of these cells *in vitro*. Despite little observable effect on FGF2 mRNA expression both 4 hour and 24 hour after exposure, C3a increased FGF2 protein secretion by fibroblasts while C5a decreased it. The established remodelling cytokines IL-4 and IL-13 clearly decreased the expression by fibroblasts of mRNA encoding remodelling mediators such as FGF2 and its receptor early after stimulation, but their effects on FGF2 protein secretion were less marked, IL-4 producing a slight net increase and IL-13 a slight reduction of FGF2 protein secretion. Neither of the anaphylatoxins significantly altered the spontaneous production by fibroblasts of IL-6 and IL-8. The precise reasons for this apparent discrepancy of the effects of the anaphylatoxins on FGF2 mRNA and protein production are not clear but would no doubt be illuminated by a more complete understanding of the time course and mechanism of secretion of this protein into the exterior.

There is abundant evidence that fibroblasts play a key role in airway inflammation and remodelling. The mucosal microenvironment composed of structural cells and highly specialised ECM is able to amplify and promote inflammation in the airways (Loubaki *et al.* 2010). In vitro and animal studies showed that interactions of inflammatory cells with structural cells play an important role in airway inflammation and remodelling (Ramos-Barbon *et al.* 2005;Plante *et al.* 2006). Structural cells, like bronchial fibroblasts, are able to produce multiple mediators that can play a role in maintaining and amplifying the

inflammatory response in asthma (Loubaki *et al.* 2010). Numerous studies have shown that fibroblasts actively define the structure of tissue microenvironments and modulate immune cell behaviour by conditioning the local cellular and cytokine microenvironment (Vancheri *et al.* 2005). Thus, fibroblasts have been shown to modulate leukocyte survival and to regulate their differentiation (Loubaki *et al.* 2010).

The demonstration here that anaphylatoxins, particularly C3a can induce fibroblast proliferation, a key feature of remodelling, is of great interest since it echoes the effects of IL-4 on fibroblasts - which is regarded as a “classic” remodelling cytokine. Interestingly TNF-α, chosen as another positive control when investigating the effects of anaphylatoxins on fibroblast proliferation, was much less effective at the concentration employed, which is surprising in view of its powerful regulation of fibroblast effector activities (Fukuda *et al.* 2003).

Substantial evidence suggests that IL-13 plays a critical role in the pathogenesis of asthma and in fibrotic elements of airway remodelling (Zhou *et al.* 2007). Although IL-13 expression has been reported to be elevated in the lungs of patients with asthma, and IL-13 has been proposed to be a major factor in asthmatic airway remodelling, evidence that IL-13 alone contributes to the airway remodelling and fibrosis in human asthma remains modest (Zhou *et al.* 2007). Some studies suggest that in contrast with murine studies, IL-13 alone has no effect on collagen expression, while actually increasing MMP production (Chen *et al.* 2005).

The Th2 cytokines are capable of effecting fibroblast proliferation. In the present study proliferation of human lung fibroblasts was increased in the presence of IL-4 and IL-13 alone, whereas the combination was less effective. The ability of

these Th2 cytokines to enhance fibroblast proliferation is consistent with previous findings. Kraft et al compared proliferation of fibroblasts from healthy controls, mild asthmatics and severe asthmatics, and found that IL-4 and IL-13, both alone significantly increased proliferation in the mild asthma group as compared with the severe asthma and healthy groups (Kraft *et al.* 2001). Proliferation after exposure to IL-13 was also significantly greater in the mild asthma group (Kraft *et al.* 2001). However, Kraft et al also noted that the combination of IL-4 and IL-13 increased fibroblast proliferation significantly in the mild asthma group as compared with the severe asthma and healthy groups (Kraft *et al.* 2001). The reason for this discrepancy with the data in the present study is not clear but could relate to ex vivo expression of IL-4/IL-13 receptors in samples obtained directly from asthmatics.

Saito and colleagues also investigated the effect of IL-4 and IL-13 on the differentiation of lung fibroblasts in the presence or absence of the Th1 cytokine IFN-γ and found that both IL-4 and IL-13 upregulated proliferation of fibroblasts (Saito *et al.* 2003). Both cytokines induced a morphological differentiation of lung fibroblasts to myofibroblasts and upregulated the expression of α-SMA. They postulated that IL-4 and IL-13 upregulate fibroblast proliferation largely by suppressing the synthesis of PGE2 (Saito *et al.* 2003). However, other signalling pathways induced by IL-4 or IL-13 might participate in cell proliferation (Saito *et al.* 2003). IL-4 and IL-13 can also activate mitogen-activated protein (MAP) kinases (Hashimoto *et al.* 2001) and this pathway can also modulate mitogenesis. They also noted that IFN-γ acts on fibroblasts and inhibits myofibroblastic differentiation induced by IL-4 and IL-13 (Saito *et al.* 2003). Numerous studies have suggested that IL-4 and IL-13 can act on fibroblasts and modulate their inflammatory responses (Hashimoto *et al.* 2001). It has also been previously reported that IFN-γ directly inhibits the production of extracellular

matrices by lung fibroblasts (Venkatesan, Roughley, & Ludwig 2002), suggesting IFN-γ to be of potential therapeutic benefit for airway remodelling (Saito *et al.* 2003).

A useful extension of the present data for future studies would be to investigate possible modulation by anaphylatoxins of surface adhesion and co-stimulatory molecules on fibroblasts. Fibroblasts express many surface adhesion and co-stimulatory molecules related to leukocyte trafficking and signalling such as ICAM-1 and VCAM-1 (Bombara *et al.* 1993). Doucet et al showed that IL-4 and IL-13 act on human lung fibroblasts to increase the expression of cell surface β1 integrin and VCAM-1 adhesion molecules which are implicated in asthma as well as in the production of IL-6 and MCP-1, two inflammatory cytokines important in the pathogenesis of allergic inflammation (Doucet *et al.* 1998a). Expression of the adhesion molecules ICAM-1 and more particularly VCAM-1 on lung fibroblasts may be important for migration of inflammatory cells through the submucosa to the airway lumen in the asthmatic inflammatory response (Spoelstra *et al.* 1999). Eosinophil infiltration into the airways, a key feature of asthma; is mediated by adhesion of eosinophils to fibroblasts through β2-integrins and their receptors (e.g. intercellular adhesion molecule-1), but VLA-4/VCAM-1 interaction could also play a role (Fukuda *et al.* 1996). Sabatini and colleagues showed that human foetal lung fibroblast cells constitutively expressed VCAM-1 and ICAM-1; while both IL-4 and TNF-α increased expression of VCAM-1, only TNF-α increased expression of ICAM-1 and increased expression of eotaxin and MCP-1 (Sabatini *et al.* 2002).

It is of great interest that C3a was observed to increase fibroblast production of FGF2 only at the protein level, whereas C5a decreased fibroblast production of FGF2 both at the protein level and at the mRNA level and increased expression

of its receptor FGFR1. The fact that C3a altered FGF2 protein but not early mRNA expression suggests that C3a exerts a late effect on FGF2 mRNA expression. Although there was no evidence of a concentration/response effect for C3a and C5a on FGFR1 expression by western blotting, the data show that HPFC do in fact express FGFR1. Existing studies suggest that FGF2 is a critical growth factor in fibrosis (Kranenburg *et al.* 2005). FGF2 has been proposed to act as a principal factor inducing fibrogenic and proliferative effects on lung fibroblasts (Selige *et al.* 2010). FGF2 regulates proliferation by interacting with its receptor and inducing mitogenesis via the MAP kinases (Thannickal *et al.* 1998). Phosphorylation of ERK1/2 has been reported to be important for mitogen-mediated proliferation (Khalil *et al.* 2005). Elevated FGF2 expression is a feature of asthma (Barnes 2003); this and other growth factors may act in concert with other cytokines such as IL-1β to induce fibrosis in asthma and other lung diseases such as Idiopathic pulmonary fibrosis (IPF) (Inoue *et al.* 2002). In addition other growth factors such as TGF-β increase FGF2 production by fibroblasts (Khalil *et al.* 2005). Fibroblasts treated with TGF-β1 show delayed (up to 2 hour) FGF2 protein synthesis (Thannickal *et al.* 1998) whereas it was demonstrated by Khalil et al that incubation of fibroblasts with TGF-β1 for as little as 1 minute could increase FGF2 release. The rapidity of these effects suggests that TGF-β1 induces the release of preformed FGF2. Up to 30 % of FGF2 synthesized by a fibroblast may be associated with the surrounding ECM (Khalil *et al.* 2005); these findings therefore suggest that a pool of FGF2 exists extracellularly and can be mobilised by growth factors such as TGF-β1 (Khalil *et al.* 2005). FGF2 and FGFR1 expression is upregulated in the remodelled airways of mice chronically challenged with allergen (Yum *et al.* 2011). Corticosteroids, on the other hand, reduce expression of both FGF2 and FGFR1 in the remodelled airway (Yum *et al.* 2011). FGF2 may influence airway remodelling not only through direct mitogenic effects on structural cells such as

smooth muscle (Bosse & Rola-Pleszczynski 2008) and fibroblasts (Strutz *et al.* 2001), but also through indirect effects on cells such as macrophages, which are induced by FGF2 to express the proremodelling cytokine TGF-β1 (Yum *et al.* 2011).

Although the anaphylatoxins did not induce IL-6 and IL-8 production by the MRC-5 fibroblast cell line, it was possible to confirm abundant production of IL-6 and IL-8 by these cells in response to the positive control TNF-α stimulus. IL-6 expression is increased in blood, BAL fluid, and the lung tissues of asthmatic patients (Doganci *et al.* 2005). Moreover, IL-6 derived from antigen-presenting cells is able to induce initial IL-4 production in naive CD4+ T cells, thereby polarizing these cells into Th2 cells (Loubaki *et al.* 2010). IL-6 plays an important role in asthma tissue remodelling (Loubaki *et al.* 2010). Multiple lines of evidence indicate that IL-6 is a good marker of fibrosis (Loubaki *et al.* 2010). Targeted over-expression of IL-6 in transgenic mice lungs leads to the development of sub-epithelial fibrosis (Kuhn *et al.* 2000). Fibrosis is also reduced in IL-6 knockout mice (Natsume *et al.* 1999). Furthermore, in tissues such as the lungs, liver and the skin, IL-6 expression has been shown to correlate with fibrosis (Smith *et al.* 1998;Kayano & Okita 2000;Sato, Hasegawa, & Takehara 2001). Loubaki et al showed that the contact between T cells and bronchial fibroblasts led to a significant increase in IL-6 production (Loubaki *et al.* 2010). IL-8 is a neutrophilic chemotactic factor that is involved in host inflammatory responses and is known to be synthesized by many different cell types, including fibroblasts (Amenomori *et al.* 2010). It attracts neutrophils to small airways and lung parenchyma, leading to tissue inflammation and destruction. Concordant with the experiments described here, Zhang and colleagues demonstrated TNF-α-induced induction of IL-6 and IL-8 in lung fibroblasts by ELISA (Zhang, Wu, & Qu 2011).

Doucet and colleagues found that human lung fibroblasts derived from either foetal or adult tissue as well as from a Th2-type stromal reaction produced IL-6, IL-8, IL-11, and MCP-1 as assayed by ELISA, whilst GM-CSF was produced in foetal fibroblasts only; eotaxin transcripts were also present in all three types of fibroblasts (Doucet *et al.* 1998a).

In summary, through their effects in increasing fibroblast proliferation and their production of key remodelling mediators, anaphylatoxins clearly have a potential role to play in remodelling processes in the human asthmatic airways. As with all inflammatory processes, there is considerable overlap of these effects with those of other mediators, particularly the Th2 cytokines IL-4 and IL-13, although evidence has been presented in this chapter that the “fine” functions of all these mediators can be distinguished. Further studies are essential to broaden and understand these relationships.

Chapter 6: Airway Smooth Muscle Cells

6.1 Introduction

Smooth muscle cells line the airways throughout the entire bronchial tree proximal to the terminal bronchioles. In the trachea, parallel bundles of trachealis muscle are attached to the cartilage rings. The muscle, found on the posterior side of the trachea, is an ideal source for sampling ASM due to the neat arrangement of parallel bundles, perpendicular to the long axis of the trachea (Amrani, Chen, & Panettieri 2000). Further down the bronchial tree, the ASM encircles the airways in a helical manner suggesting that contraction would both narrow the airways and shorten their length (Amrani, Chen, & Panettieri 2000). The airways of asthmatics are known to have an increased mass of ASM. Whether this is due to hypertrophy (an increase in size of airway smooth muscle cells) or hyperplasia (an increase in the number of airway smooth muscle cells) or both is still unclear (Ebina *et al.* 1993).

In 1922, Huber and Koessler described, for the first time, an increase in ASM tissue in the airways of asthmatics (Huber & Koessler 1922). Since then, enlargement of peribronchial ASM tissue has become a histopathological signature of the disease (Hirst *et al.* 2004). By generating more force, this increased mass of ASM is generally thought to contribute to AHR (Hirst *et al.* 2004).

There has been much speculation about the possible physiological function of ASM. Human ASM cells are involved in the pathogenesis of asthma, because these cells contribute to AHR and airway obstruction (Bloemen *et al.* 2007). ASM is known to proliferate in response to numerous growth factors and mediators that are released during allergic airway inflammation both *in vitro* and

in vivo (Gosens *et al.* 2008). In addition, smooth muscle hypertrophy and hyperplasia are accepted features of airways remodelling.

Many inflammatory mediators increased in asthmatic airways induce airway smooth muscle proliferation *in vitro* (Moynihan et al. 2008). Classical mediators, such as histamine, thromboxane, and leukotrienes are potent airway smooth muscle constrictors and potential smooth muscle mitogens (Moynihan *et al.* 2008). Pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are also increased in asthmatic airways and there is some evidence that they enhance smooth muscle proliferation. Th2 cytokine receptors, such as IL-4 and 13 receptors, are also expressed on airway smooth muscle cells (Moynihan *et al.* 2008).

An important aspect of interaction between airway inflammation and airway smooth muscle is that airway smooth muscle itself could be a source of mediators of airway inflammation and remodelling (Moynihan *et al.* 2008). Studies have revealed that airway smooth muscle cells release several mediators including cytokines (such as GM-CSF, IL-2, -5, -6, -11, -12, -13, IFN-γ), chemokines (such as eotaxin, RANTES, IL-8, MCP-1, -2, -3, TARC), growth factors (PDGF, IGF, SCF, VEGF) and inflammatory mediators in the airways (PGE2, PLA2) (Howarth *et al.* 2004). Several chemokines are also produced by human ASM cells *in vitro* upon stimulation with cytokines (IL-4, IL-13, IL-1β, TNF-α, TGF-β and PAF), including CCL5, CCL7, CCL11, CCL13 and CXCL8 (Bloemen *et al.* 2007). For instance, a marked increase in CCL11 immunoreactivity in ASM was seen in an immunological study of the airways from patients with asthma (Black & Johnson 2002). These chemokines are involved in the recruitment of inflammatory cells into the lung and promote the

upregulation of cell surface adhesion molecules (Bloemen *et al.* 2007). Furthermore GM-CSF, which is an important stimulator of maturation, activation and survival of several inflammatory cells, is produced by ASM cells when stimulated by a combination of IL-1β and TNF-α (Oltmanns *et al.* 2003). Bradykinin can increase IL-6, CXCL8 and PGE2 release (Pang & Knox 1998). Therefore, airway smooth muscle cells might be a source of mediators of airway inflammation, and may modulate autocrine proliferative responses (Moynihan *et al.* 2008).

Moynihan and colleagues examined cultured human (H)ASM cells for the expression of IL-13 receptor subunits (Moynihan *et al.* 2008). They found that the receptor subunits were expressed on HASM cells, and that pre-treatment with IL-4 and IL-13, but not IFN-γ induced desensitization of the HASM cells to IL-13 as measured by eotaxin secretion. The mechanism of IL-4 and IL-13 induced desensitization did not appear to involve downregulation of receptor expression (Moynihan *et al.* 2008).

Animal models demonstrate a role for IL-13 in the development of AHR; overexpression of IL-13 in the murine lung induces a phenotype similar to human asthma, with excess mucus production, goblet cell hyperplasia and smooth muscle hypertrophy (Zhu *et al.* 1999). IL-13 and IL-4/IL-13 knockout mice are protected from allergen induced AHR (Leigh *et al.* 2004). Interferon-γ may counteract the actions of IL-4 and IL-13 in some circumstances. IFN-γ knockout mice have been shown to have augmented Th2 responses and IFN-γ knockout mice have enhanced AHR following allergen challenge that is restored by administration of recombinant IFN-γ (Yoshida *et al.* 2002).

Although there are many studies on airway smooth muscle cells and their role in asthma, there are few in the context of the complement system together with Th2 cytokines and growth factors. Here it was hypothesised that complement components, particularly the anaphylatoxins are capable of increasing proliferation of human airway smooth muscle cells and their production of remodelling mediators.

The specific aims of the studies described in this chapter were to:

1. Assess the expression of C3aR and C5aR on human smooth muscle cells;
2. Assess the proliferative response of smooth muscle cells to stimulation with anaphylatoxins and Th2 cytokines;
3. Examine C3a and C5a-stimulated production of important remodelling mediators (such as growth factors) by smooth muscle cells at the mRNA and protein level, comparing with Th2-type cytokine-stimulated production as a positive control.

6.2 Results

6.2.1 Expression of complement receptors on primary cultured human airway smooth muscle cells

Examination of these cells by light microscopy revealed an elongated, linear and spindle shaped morphology with prominent nuclei usually in the centre. Immunohistochemistry demonstrated that C3aR and C5aR were both expressed on airway human smooth muscle cells (Figure 6.1). This pattern of staining was observed consistently in all smooth muscle cell cultures that were stained in this fashion.

6.2.2 Proliferation of airway smooth muscle cells

The effect of the anaphylatoxins and inflammatory cytokines on the proliferation of human smooth muscle cells (HSMC) was studied. IL-4 induced statistically significant proliferation, whereas IL-13 alone did not. However, the combination further induced proliferation to a mean of 135.37 % of the baseline rate (Figure 6.2A). C3a at a relatively high concentration of 10^{-7} M also significantly increased proliferation (Figure 6.2B) but not at lower concentrations. C5a in the concentration range of 10^{-9}-10^{-7} M exerted no significant effect (Figure 6.2C). The concentration-response curves were blunted by the inherent variability of individual responses.

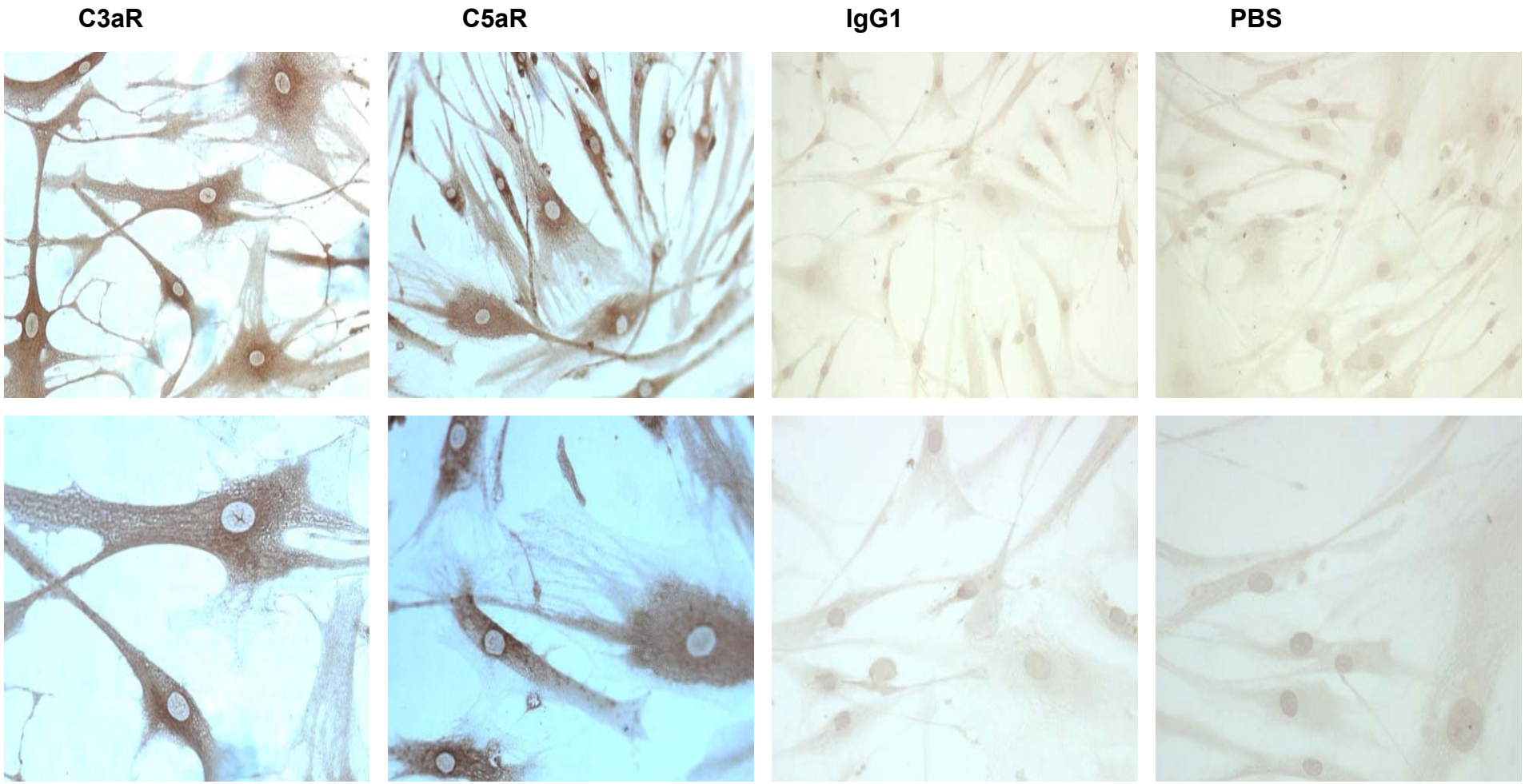

Figure 6.1 Expression of C3aR and C5aR on HSMC by immunocytochemistry. HSMC cultured as described in Chapter 2: Materials & Methods, section 2.1.3.1 were immunostained for the anaphylatoxin receptors C3aR and C5aR (ICC detailed in Chapter 2: Materials & Methods, section 2.2.5). Immunostaining with an isotype matched control primary antibody (IgG1) and omitting the primary antibody (PBS) were used as negative controls. The results shown are representative of 3 experiments. Magnification: top panel x10; bottom panel x20.

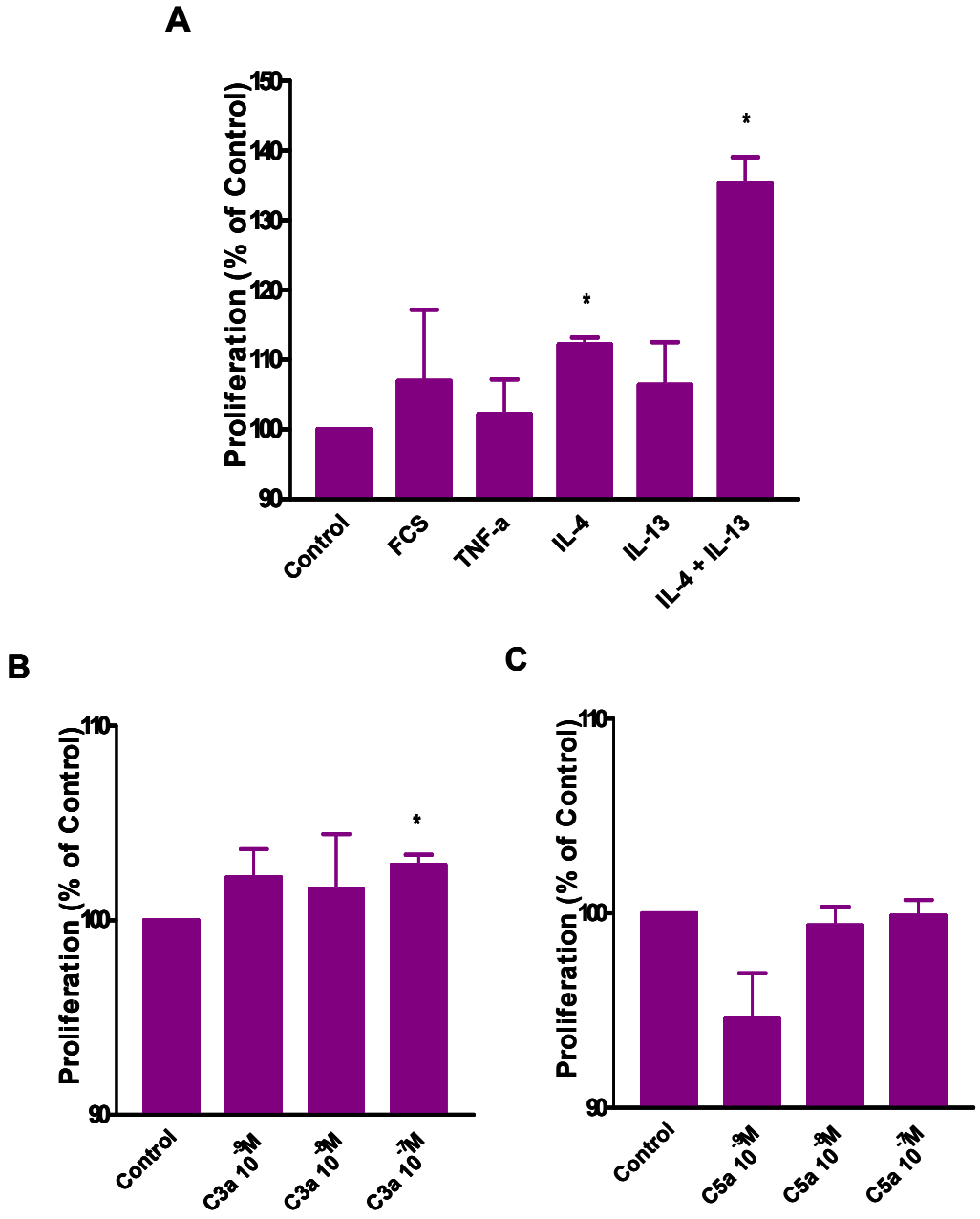

Figure 6.2 Effects of cytokines, C3a and C5a on proliferation of HSMC. Cells were exposed to conditions for 48 h. Conditions included treatment with A) FCS (2%) and inflammatory cytokines (at 10 ng/ml) and B) C3a and C) C5a at different concentrations (10^{-9}-10^{-7} M) (refer to Chapter 2: section 2.3 for details of proliferation method). Data are expressed as mean ± SEM of the response of 3 independent MTS assay experiments, expressed as a percentage of the control cells set to 100%. * $p<0.05$ for induction of proliferation vs control (Student's t-test).

6.2.3 FGF gene expression in airway smooth muscle cells

Cultured HSMC were screened for the expression of FGF ligands and receptors at the mRNA level (Figure 6.3). The particular products analysed were defined from a more preliminary screen. Gene expression of FGF2, FGF17 and FGFR4 in HSMC was measured at 4 and 24 hours following stimulation and analysed by qPCR. TNF-α stimulation of HSMC at the concentrations employed had no significant effect on the expression of mRNA encoding the FGF ligands and receptor (Figure 6.3A). IL-4-stimulated HSMC showed an early (4 h) upregulation in the expression of mRNA encoding FGF17, which was downregulated at 24 hours compared to control (Figure 6.3B). Similarly, IL-13 stimulation showed a late (24 h) downregulatory response in the expression of FGF17 mRNA; there was a trend for earlier upregulation, which was not significant because of large variation between the samples (Figure 6.3C). Both Th2 cytokines had no effect on FGF2 and FGFR4 expression at the mRNA level. C3a considerably reduced expression of mRNA encoding FGF2 both early and late after stimulation; mRNA encoding FGF17 showed late suppression by C3a (Figure 6.3D). A similar late response was observed with C5a, which reduced HSMC expression of mRNA encoding FGF2 and FGF17 to 50 % or less of the basal level (Figure 6.3E).

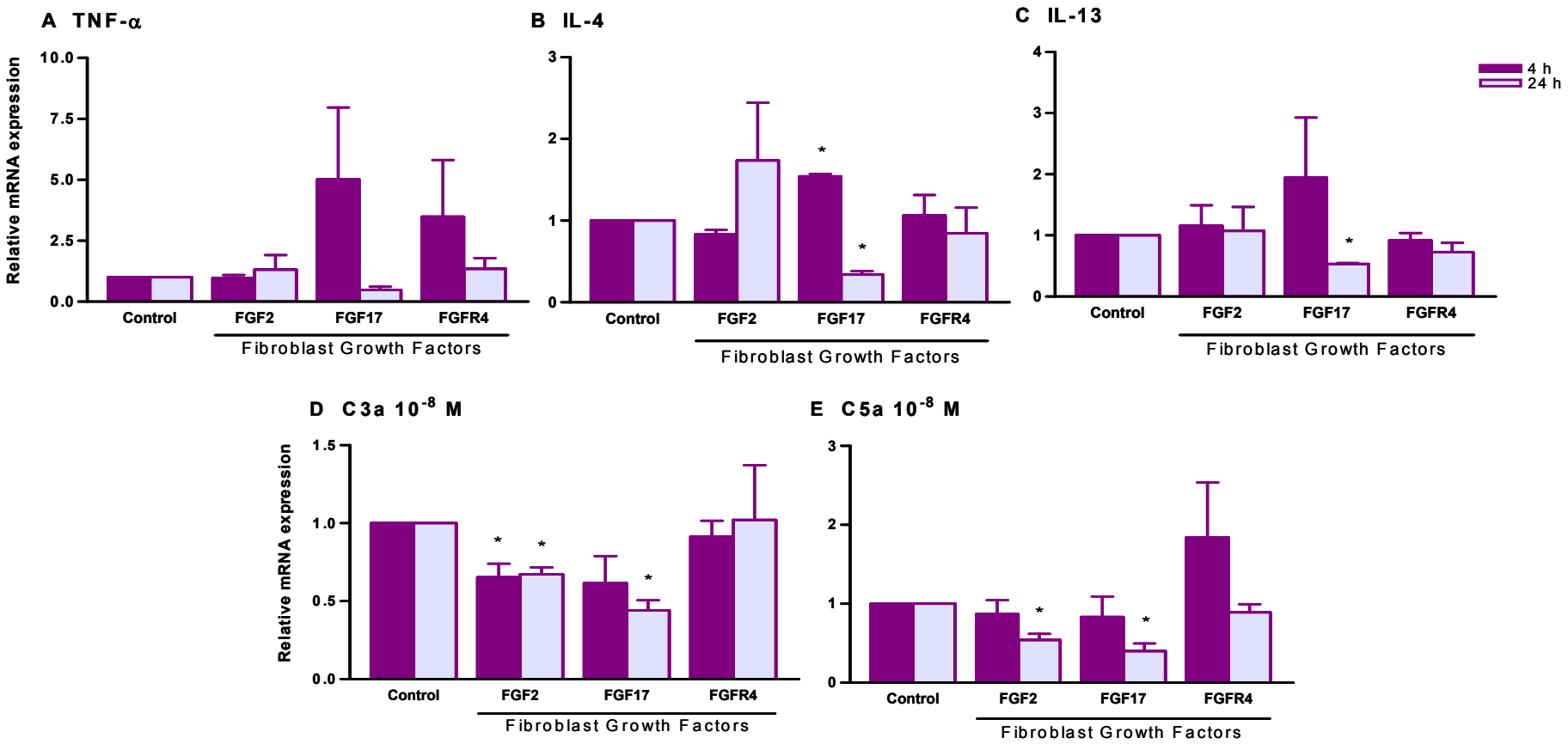

Figure 6.3 Gene expression (relative to control cultures) encoding FGF2, FGF17 and FGFR4 mRNA in HSMC by real time qPCR. Cells were treated with A) TNF-α (10 ng/ml) B) IL-4 (10 ng/ml) C) IL-13 (10 ng/ml) D) C3a 10^{-8} M E) C5a 10^{-8} M for 4 and 24 h to measure FGF mRNA levels (qPCR detailed in Chapter 2: Materials & Methods, section 2.4.3). Data are expressed as mean ± SEM of the response of 3 independent experiments normalized relative to 18s mRNA. Statistical significance was assessed by Student's t-test, * $p<0.05$ vs. control cells at the same time point.

6.2.4 FGF2 protein expression by smooth muscle cells

The effects of C3a, C5a and cytokines (at 10 ng/ml) on the expression and release of FGF2 were studied in HSMC by ELISA (Figure 6.4). HSMC spontaneously released FGF2. However, production was suppressed to around half of the basal level under the conditions employed by TNF-α, IL-4, IL-13 as well as the anaphylatoxins. Both C3a and C5a inhibited FGF2 production at all physiological concentrations.

6.2.5 Detection of FGFR1 in smooth muscle cells

The effects of C3a, C5a and the Th2 cytokines (IL-4 and IL-13 at 10 ng/ml) on the expression of FGFR1 was investigated by Western blotting (see Chapter 2: section 2.5.4 for western blotting method). Neither the anaphylatoxins nor the cytokines were found to induce detectable expression of this growth factor receptor (data not shown).

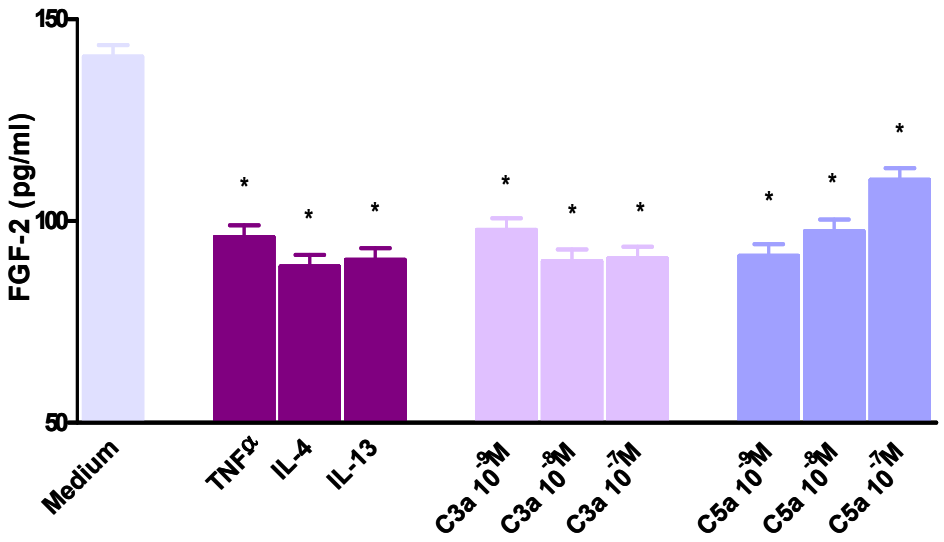

Figure 6.4 Effects of inflammatory cytokines, C3a and C5a on FGF2 expression by HSMC. Cells were cultured and stimulated for 48 h with various concentrations of C3a and C5a ranging from 10^{-9} M to 10^{-7} M and Th2 cytokines and TNF-α at 10 ng/ml. Supernatants were harvested and FGF2 expression was assessed by ELISA as described in Chapter 2: Materials & Methods, section 2.5.3. Data are expressed as mean ± SEM of the response of 3 independent experiments, * $p<0.05$ compared with medium control (Student's t-test).

6.3 Discussion

In this chapter it has been shown that primary cultured human airway smooth muscle cells express anaphylatoxin receptors, and that complement components have the propensity to regulate smooth muscle cellular proliferation and the production of key remodelling mediators.

ASM proliferation is of functional relevance in asthma because smooth muscle hyperplasia is thought to be a fundamental feature of airway remodelling and asthma pathogenesis (Takeda *et al.* 2006). A study by Lambert and colleagues suggested that if airway smooth muscle contractile properties are maintained, increased airway smooth muscle mass in asthma may be the single most important contributing factor to exaggerated airway narrowing (Lambert *et al.* 1993). Thus it is critical to elucidate the factors that may modify the proliferation of human airway smooth muscle cells.

Nevertheless it is somewhat controversial whether smooth muscle proliferation does in fact occur in human asthma (Benayoun *et al.* 2003;Woodruff *et al.* 2004). An *ex vivo* assessment of proliferative markers showed no evidence of increased basal proliferation of myofibroblasts cultured from asthmatic biopsies (Ward *et al.* 2008). Immunostaining for cyclin D1, a marker of proliferation, revealed that although cyclin D1 immunoreactive cells increased with asthma severity, with a 3.5-fold higher median value for severe asthmatics compared with non-asthmatics (Ward *et al.* 2008), cyclin D1 immunoreactivity was not detected in the smooth muscle bundles but was restricted to the epithelium and subepithelial connective tissue areas of the histological sections (Ward *et al.* 2008). A possible explanation for this discrepancy is that detection of asthma-

related changes in proliferation may be less apparent when cells are maintained in culture isolated from the asthmatic micro-environment of the intact airway wall. On the other hand there are reports of enhanced spontaneous proliferation of human ASM cells from asthmatics compared with controls (Black & Johnson 2002).

IL-4 and IL-13 have previously been shown to affect ASM proliferation either directly or by altering ASM responsiveness to other mitogens (Hawker *et al.* 1998;Hirst *et al.* 2004;Faffe *et al.* 2006). In our hands and, albeit at a single physiological concentration, IL-4 but not IL-13 alone increased smooth muscle proliferation, but they showed an impressive effect in combination. Bosse and colleagues reported a similar effect of IL-4 and IL-13 on ASM cell proliferation, supporting a potential role for both cytokines in airway remodelling (Bosse *et al.* 2008). Other studies have on the other hand suggested that IL-4 suppresses proliferation of ASM cells (Faffe *et al.* 2006).

IL-4Rα as well as IL-13RαI and IL-13RαII have all been reported to be constitutively expressed in human ASM cells (Tomlinson, Wilson, & Stewart 1994). However, there are limited data on the signalling pathway of IL-4 after it binds to its receptor (Hirst *et al.* 2004). In one study on cultured human ASM cells, both IL-4 and IL-13 activated IL-4Rα and induced phosphorylation of its signal transducer and activation of transcription-6 (STAT6), p42/p44 ERK and p38 MAP kinase (Tomlinson, Wilson, & Stewart 1994). However, since ERK and p38 MAP kinase are known to be important intracellular pathways for cell proliferation, it is unlikely that IL-4 suppresses ASM cell proliferation through them (Hirst *et al.* 2004). It has been suggested that IL-4 decreases ASM cell proliferation by decreasing cyclin D1 protein expression rather than a c-AMP dependent mechanism (Ebina *et al.* 1993) or through STAT6 activation.

IL-13 has diverse effects on HASM cells that may have important implications for the pathobiology of asthma (Moynihan *et al.* 2008). However the responses of HASM cells to the presence of IL-13 is likely to be conditioned by the prevalent cytokine milieu (Moynihan *et al.* 2008). Exposure of HASM cells to IFN-γ reduced the effects of IL-13 on STAT6 activation but did not appear to have much effect on calcium responses to histamine and on eotaxin secretion, which are important measures of contractile and secretory properties of HASM (Moynihan *et al.* 2008).

The new data reported in this study suggest that C3a, at high physiological concentrations, but not C5a can alter smooth muscle proliferation. Thus the anaphylatoxins must be added to the list of mediators potentially able to influence HASM proliferation.

FGF2 features highly in the remodelling literature. It is the only member of the FGF family known to be upregulated in asthma and has been reported to increase ASM proliferation alone or in synergy with IL-4/IL-13 (Bosse *et al.* 2008). The new data in the present study suggest that C3a and C5a might antagonise this. In our study the same concentrations of IL-4 and IL-13 (both at 10 ng/ml) were used as in Bosse et al's study. In contrast to the present findings, Bosse et al did not find that IL-4 at the same concentration as in the present study increased BSMC proliferation, but the IL-13 finding was in accordance in the sense that IL-13, at different concentrations, including at 10 ng/ml, did not affect BSMC proliferation when administered alone but altered the effects of other cytokines (Bosse *et al.* 2008). On the other hand a study by Hawker and colleagues suggested that IL-4 reduced the mitogenic effect of FGF2 (Hawker *et al.* 1998). IL-4 is just one of the cytokines that has been shown

to either promote or inhibit airway smooth muscle proliferation *in vitro*. The conflicting results could partly reflect the methods employed to measure cellular proliferation. TNF-α also has a small stimulatory effect on human airway smooth muscle proliferation (Hawker *et al.* 1998). In addition, TNF-α, like IL-4, can inhibit mitogen-induced proliferation of human airway smooth muscle cells (Stewart *et al.* 1995). In our hands TNF-α had no significant effect on the growth factors tested. The data might have been clearer had the numbers of experiments been increased.

FGF2 has also been reported to enhance the proliferative effect of TGF-β on ASM (Bosse *et al.* 2006) so C3a may also influence this effect. TGF-β, known as a growth factor inducing cell differentiation and fibrosis, is well known to be over-expressed in asthma. In addition, eosinophils, a major player in allergic airway inflammation, appear to be a major source of TGF-β (Ohno *et al.* 1996).

Preliminary screening of the FGFs and their receptors at the mRNA level showed that only FGF2, FGF17 and FGFR4 appeared to be regulated by anaphylatoxin and Th2 cytokines in airway smooth muscle cells. C3a and C5a reduced FGF2 and FGF17 expression at the mRNA level and FGF2 at the protein level. This suggests that C3a and C5a may be able to inhibit remodelling. Inhibition of FGF17 by C3a and C5a might be important as this growth factor is fairly new in the context of asthma and remodelling. FGF17 is a molecule analogous to FGF8 (which was originally isolated as an androgen-induced growth factor (AIGF)) and has been found in the smooth muscle of major artery walls. Both FGF2 and FGF17 are capable of binding FGFR4. The precise repertoire of FGF receptors on human ASM is not well defined but could include FGFR1c. FGF2 seems to possess a greater binding specificity for the IIIc splice form of FGFR1 to R3 and with FGFR4 but also demonstrates some

activity toward the FGFR1b-splice form (Ornitz *et al.* 1996). This is unlike FGF1, which appears to be a universal FGFR ligand. Kranenburg and coworkers have detected FGFR1 in vascular smooth-muscle cells, ASM cells and the airway epithelium (Kranenburg *et al.* 2002). It is thus believed that the FGFR1c is expressed on ASM cells and might be responsible for the FGF2-mediated biological effects.

Collectively, the published studies regarding the expression of FGF2 and its receptor in asthmatic airways consistently reported an increased expression of FGF2, although the cellular origin of this FGF2 and the precise receptor(s) responsible for transducing its biological effects in ASM remain to be clarified (Bosse & Rola-Pleszczynski 2008).

By upregulating (at least modestly in the case of C3a) ASM proliferation and suppressing FGF2 production anaphylatoxins could be contributing significantly to the functional activities of these mediators in remodelling. The research outlined in this chapter suggests that anaphylatoxins, their receptors, as well as Th2 and other cytokines may exert direct effects and have an impact on smooth muscle cells and thus could be involved in remodelling of the human asthmatic airway.

Chapter 7: Endothelial Cells

7.1 Introduction

Common to all vessels of the vasculature is the endothelium, which is a single layered sheet of squamous, polarised cells that are primarily responsible for all signalling and transportation from blood to tissue and vice versa (Cines *et al.* 1998). The endothelium plays an important role in inflammation by actively participating in its initiation and propagation (Albrecht *et al.* 2004).

Activation of the endothelium by lipopolysaccharide (LPS) or TNF-α is known to promote the expression of adhesion proteins (eg, E-selectin, ICAM-1, VCAM-1) that initiate adhesive interactions with blood leukocytes (Albrecht *et al.* 2004). These adhesive interactions may cause leukocytes to encounter chemokines on the endothelial surface, which results in leukocyte activation and engagement of leukocyte integrins (LFA-1 and Mac-1, VLA-4) with their counter receptors (ICAM-1 and VCAM-1) on endothelial cells (Albrecht *et al.* 2004). The resulting firm attachments enable leukocytes to move through endothelial junctions. Thus, physical interactions between the endothelium and leukocytes are part of the inflammatory response, whereas additional contributions are provided by leukocytic receptors, which ligate inflammatory mediators such as C5a, TNF-α, and CXC chemokines (Albrecht *et al.* 2004).

Endothelial cells are important in angiogenesis and neovascularisation, key processes in remodelling in asthma and many other pathological processes e.g. tumour metastasis. As the body ages, so does the vasculature and ageing of the vasculature is accompanied by a number of changes such as reduced vasoregulation, delayed and/or altered angiogenesis, reduced repair capacity and altered blood vessel composition (Boisen *et al.* 2010).

Different types of endothelial cells have been isolated and cultured to understand the biology and pathobiology of the vasculature and the angiogenic response. The most widely used type isolated from human umbilical vein (named HUVEC) shows a characteristic cobblestone shape when cultured, although cell borders are indistinct and hard to visualize (Jaffe *et al.* 1973). In addition to HUVEC, primary cultures of human pulmonary microvascular endothelial cells (HPMEC) isolated from single normal human donors were also studied.

Very few studies have been performed on endothelial cells in the context of asthma and the complement system. It was hypothesised that C3a and C5a play a role in effecting airway remodelling by acting on endothelial cells to induce remodelling changes or production of remodelling mediators.

The specific aims of the studies described in this chapter were to:

1. Assess the expression of C3aR and C5aR on endothelial cells;
2. Assess the proliferative response of endothelial cells to stimulation with anaphylatoxins and Th2 cytokines;
3. Examine C3a and C5a-stimulated production of IL-6 and IL-8 by endothelial cells at the mRNA and protein level, comparing with Th2-type cytokine-stimulated production as a positive control.

7.2 Results

7.2.1 Expression of complement receptors on endothelial cells

Immunocytochemistry demonstrated that both HUVEC (Figure 7.1) and HPMEC (Figure 7.2) expressed both C3aR and C5aR.

7.2.2 Proliferation of endothelial cells

Effects of anaphylatoxins and cytokines on endothelial cellular proliferation were studied. While both IL-4 and IL-13 at 10 ng/ml did not induce significant HPMEC proliferation alone, the combination of both did (Figure 7.3A). TNF-α at this single concentration was similarly ineffective. FCS (2%), present in the anaphylatoxin solutions, also increased HPMEC proliferation. C3a at a range of physiological concentrations significantly induced increased proliferation although there was little evidence of a concentration response effect (Figure 7.3B). In contrast, C5a did not have any significant effect on the proliferation of HPMEC (Figure 7.3C), although the considerable between experiment variability may have precluded statistical significance.

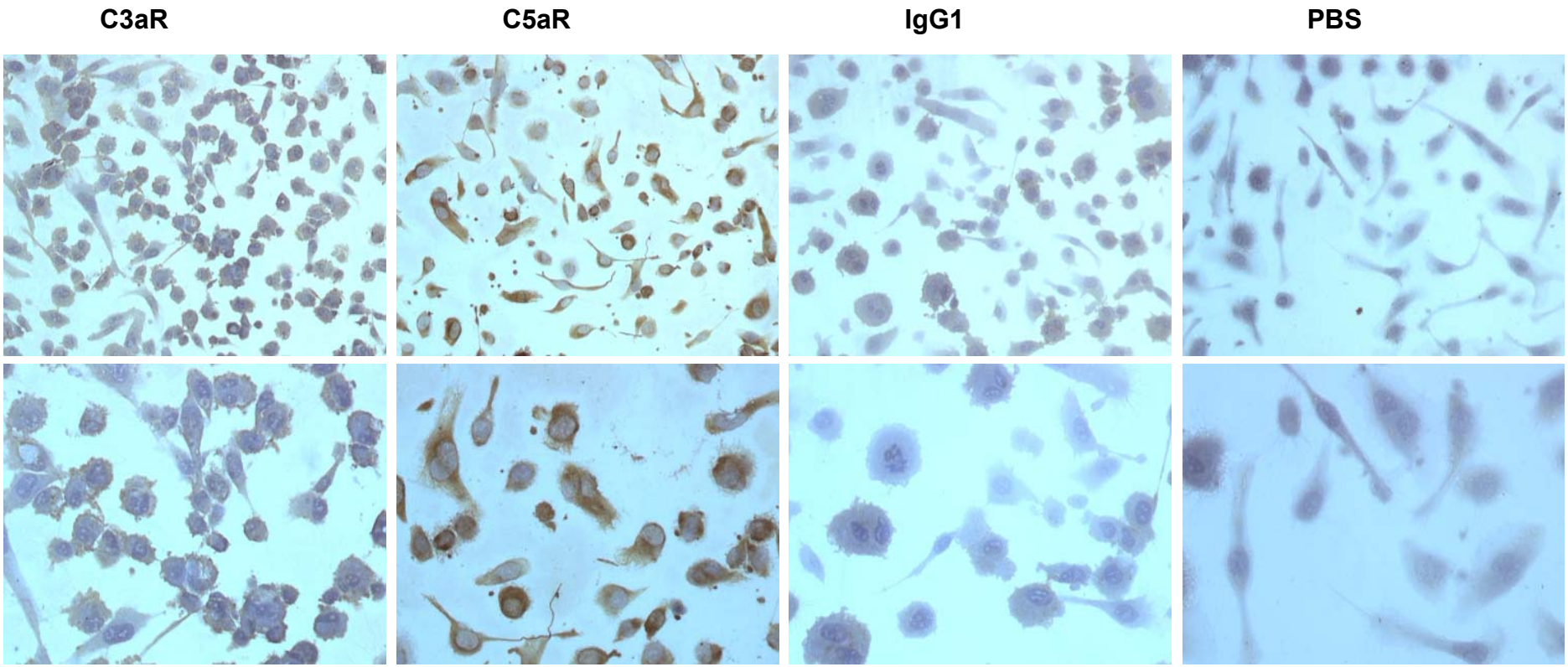

Figure 7.1 Expression of C3aR and C5aR on HUVEC by immunocytochemistry. HUVEC cultured as described in Chapter 2: Materials & Methods, section 2.1.4.1 were immunostained for the anaphylatoxin receptors C3aR and C5aR (ICC detailed in chapter 2: Methods; section 2.2.5). Immunostaining with an isotype matched control primary antibody (IgG1) and omitting the primary antibody (PBS) were used as negative controls. The results shown are representative of 3 experiments. Magnification: top panel x10; bottom panel x20.

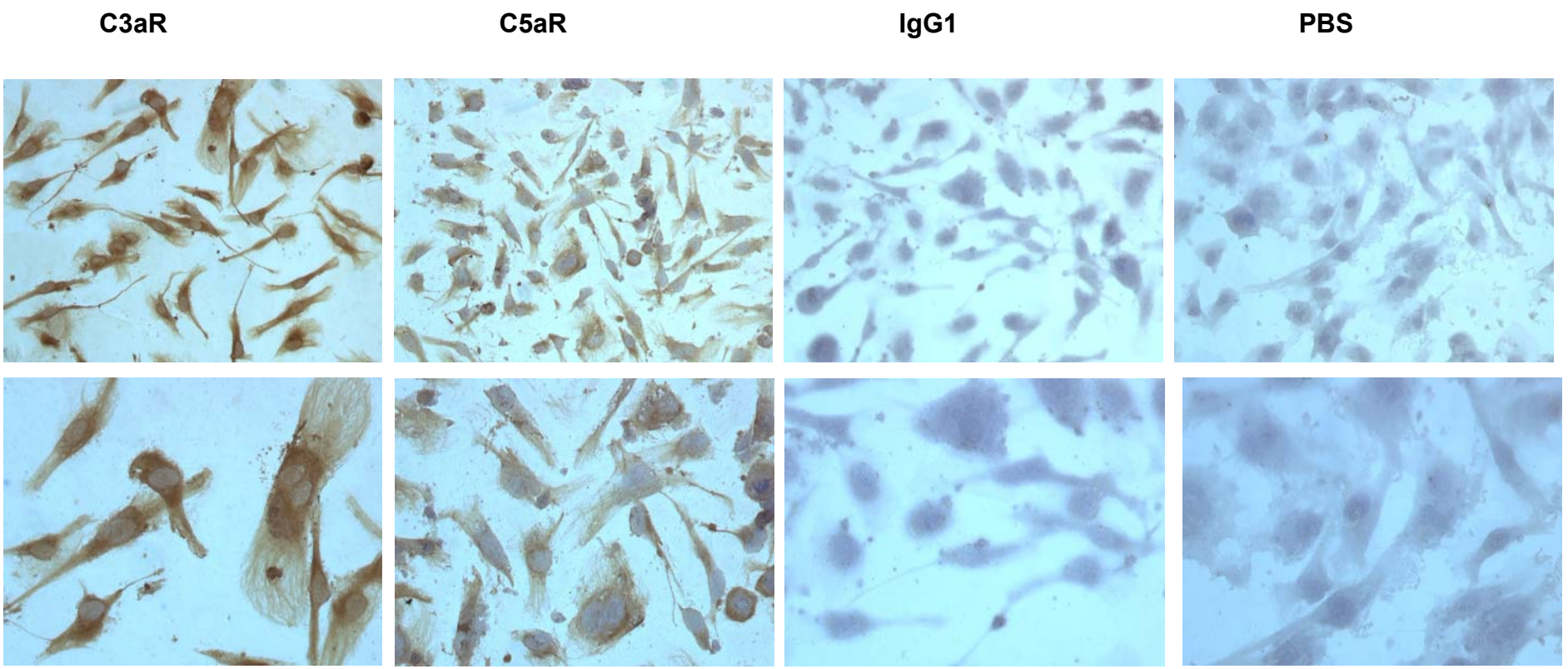

Figure 7.2 Expression of C3aR and C5aR on HPMEC by immunocytochemistry. HPMEC cultured as described in Chapter 2: Materials & Methods, section 2.1.4.2 were immunostained for the anaphylatoxin receptors C3aR and C5aR (ICC detailed in chapter 2: Methods; section 2.2.5). Immunostaining with an isotype matched control primary antibody (IgG1) and omitting the primary antibody (PBS) were used as negative controls. The results shown are representative of 3 experiments. Magnification: top panel x10; bottom panel x20.

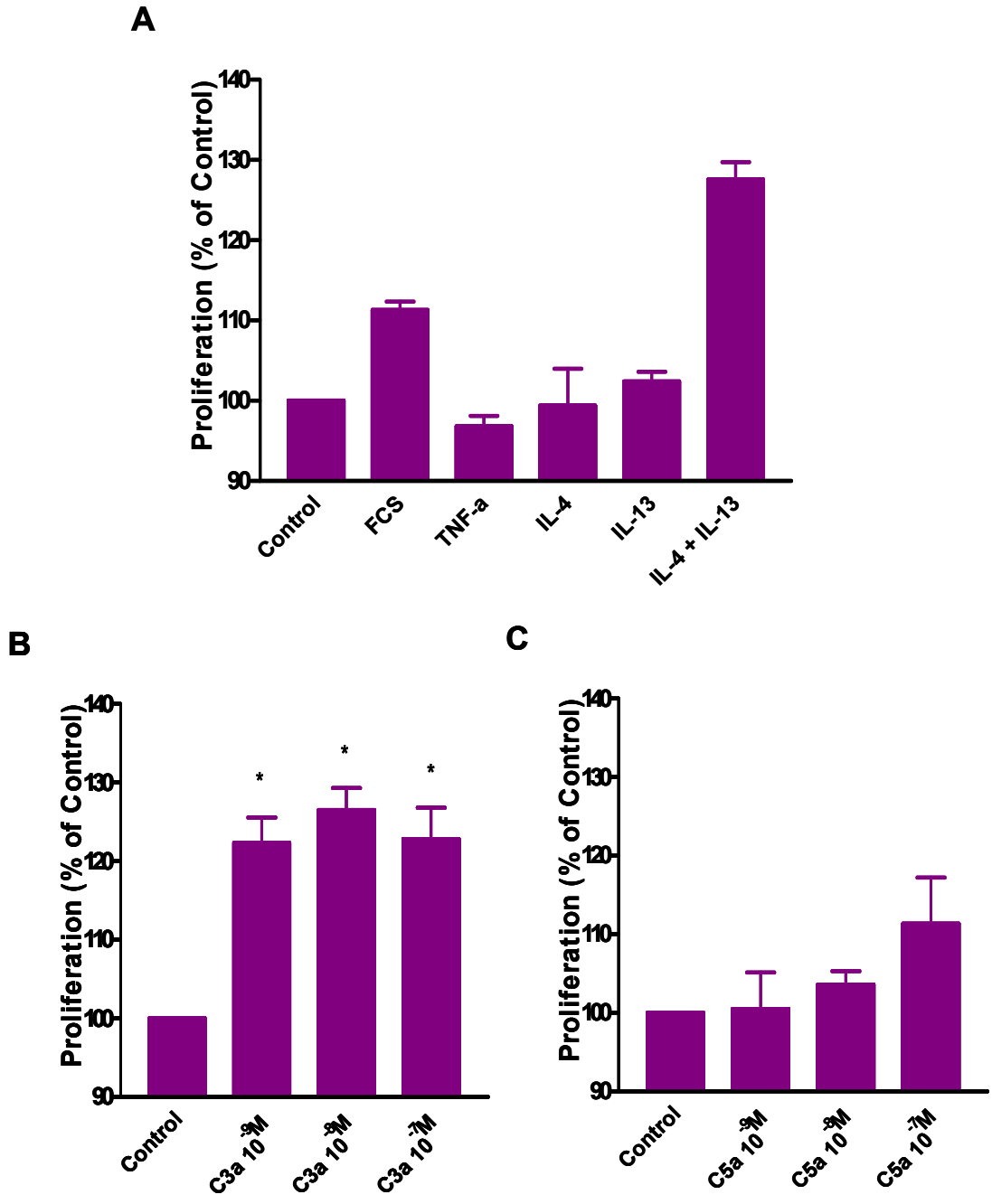

Figure 7.3 Effects of inflammatory cytokines, C3a and C5a on proliferation of HPMEC. Cells were exposed to conditions for 48 h. Conditions included treatment with A) FCS (2%) and inflammatory cytokines (at 10 ng/ml) and B) C3a and C) C5a at different concentrations (10^{-9}-10^{-7} M) (refer to Chapter 2: section 2.3 for details of proliferation method). Data are expressed as mean ± SEM of the response of 3 independent MTS assay experiments, expressed as a percentage of the control cells set to 100%, * $p<0.05$ for induction of proliferation vs control (Student's t-test).

7.2.3 FGF gene expression in endothelial cells

The potential of anaphylatoxins and Th2 cytokines to induce the expression of pro-fibrotic genes in endothelial cells at the mRNA level was assessed by qPCR (Figure 7.4). The particular growth factors analysed were defined from a more preliminary screen (see discussion). Expression of mRNA encoding FGF2, FGFR1, FGFR4 and FGFRL1 in HPMEC were measured at 4 and 24 hour following stimulation. Both IL-4 and IL-13 upregulated expression of mRNA encoding FGF2 and FGFR4 early (4 h) after stimulation, with both early and late (24 h) significant downregulation of mRNA encoding FGFRL1. Both cytokines also upregulated expression of mRNA encoding FGF2 late (24 h) after stimulation. In addition, IL-13 significantly upregulated FGFR1 mRNA expression late (24 h) after stimulation. TNF-α did not alter expression of any of these mRNA species in the endothelial cells. C3a at a concentration of 10^{-8} M upregulated FGF2 mRNA early (4 h) after exposure. There was a trend for an effect of C5a particularly on FGF2 and FGFR4 upregulation but the variability of the data precluded statistical significance.

7.2.4 IL-6 and IL-8 protein expression by endothelial cells

The effects of C3a and C5a on the expression of IL-6 (Figure 7.5) and IL-8 (Figure 7.6) were examined in HUVEC by ELISA. HUVEC spontaneously produced low amounts of IL-6 in culture which was greatly augmented by TNF-α (used as a positive control: Figure 7.5). Spontaneous production of IL-8 by HUVEC was higher still and still clearly augmented by TNF-α (Figure 7.6). By comparison, C3a had no significant effect on IL-6 or IL-8 production at a range of physiological concentrations, whilst C5a at the lowest concentrations

employed slightly increased IL-6 (at 10^{-10} M) and IL-8 (at 10^{-10} M to 10^{-9} M) production at the later time point of 72 hours (Figures 7.5, 7.6).

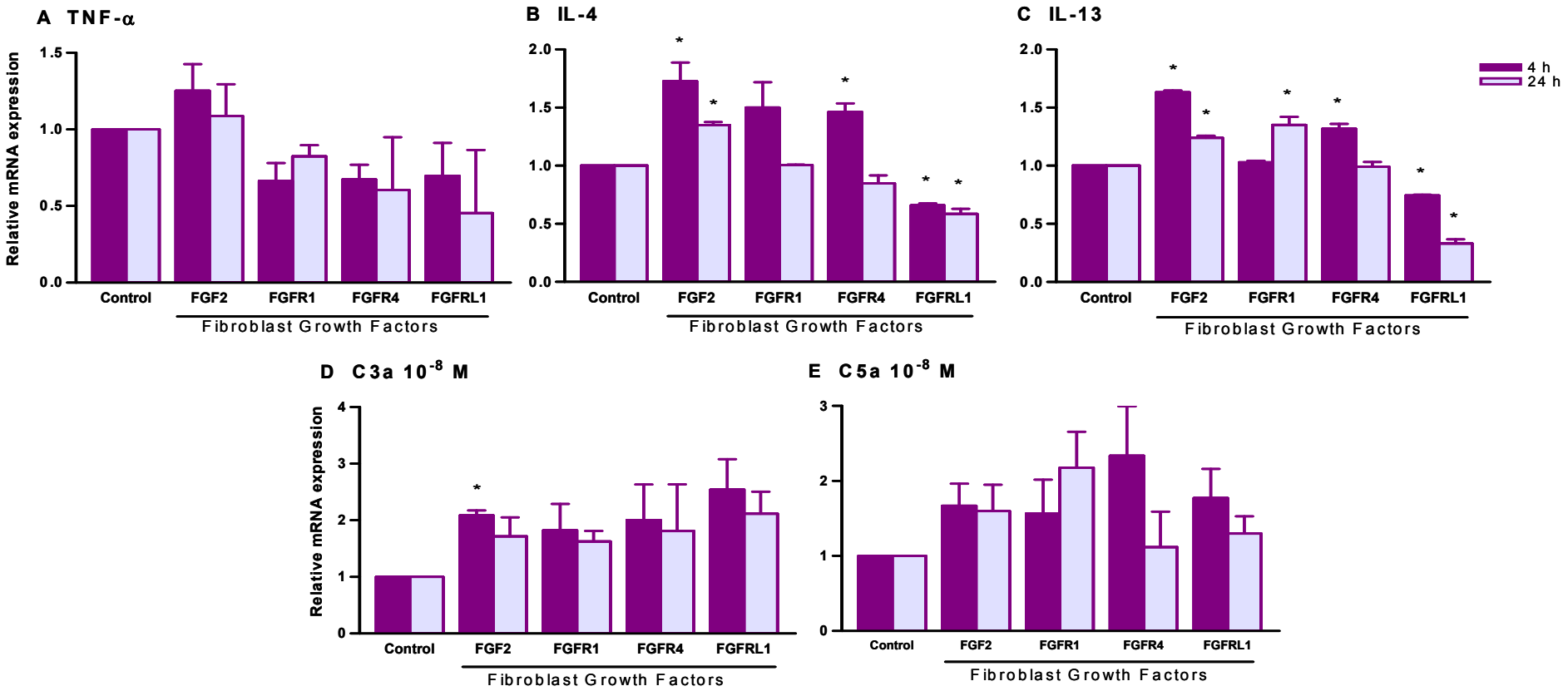

Figure 7.4 Relative expressions of FGF2, FGFR1, FGFR4 and FGFRL1 mRNA in HPMEC by real time qPCR. Cells were treated with A) TNF-α (10 ng/ml) B) IL-4 (10 ng/ml) C) IL-13 (10 ng/ml) D) C3a 10^{-8} M E) C5a 10^{-8} M for 4 and 24 h to measure FGF mRNA levels (qPCR method detailed in Chapter 2: Materials & Methods, section 2.4.3). Data are expressed as mean ± SEM of the response of 3 independent experiments normalized relative to 18s mRNA. Statistical significance was assessed by Student's t-test, * $p<0.05$ vs control cells at the same time point.

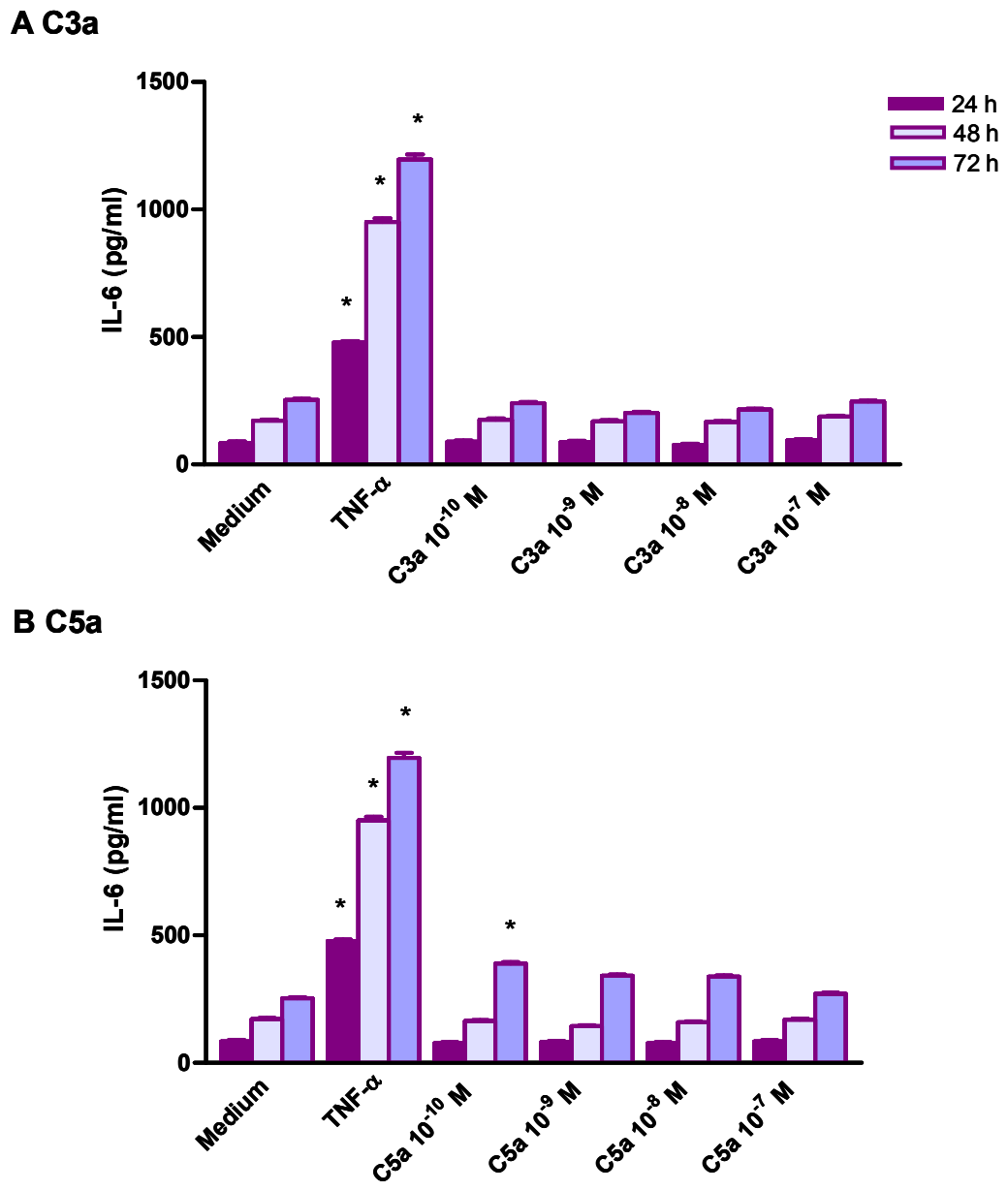

Figure 7.5 Effects of C3a and C5a on IL-6 production in HUVEC. Cells were cultured as described in Chapter 2: Materials & Methods, section 2.1.4.1 and stimulated for 24-72 h with various concentrations of A) C3a and B) C5a ranging from 10^{-10} M to 10^{-7} M, and TNF-α at 10 ng/ml as a positive control. Supernatants were harvested and IL-6 expression was measured by ELISA (refer to Chapter 2: Materials & Methods, section 2.5.3 for details of ELISA method). Data are expressed as the mean ± SEM of 3 independent experiments, * $p<0.05$ vs medium control at the same time point (Student's t-test).

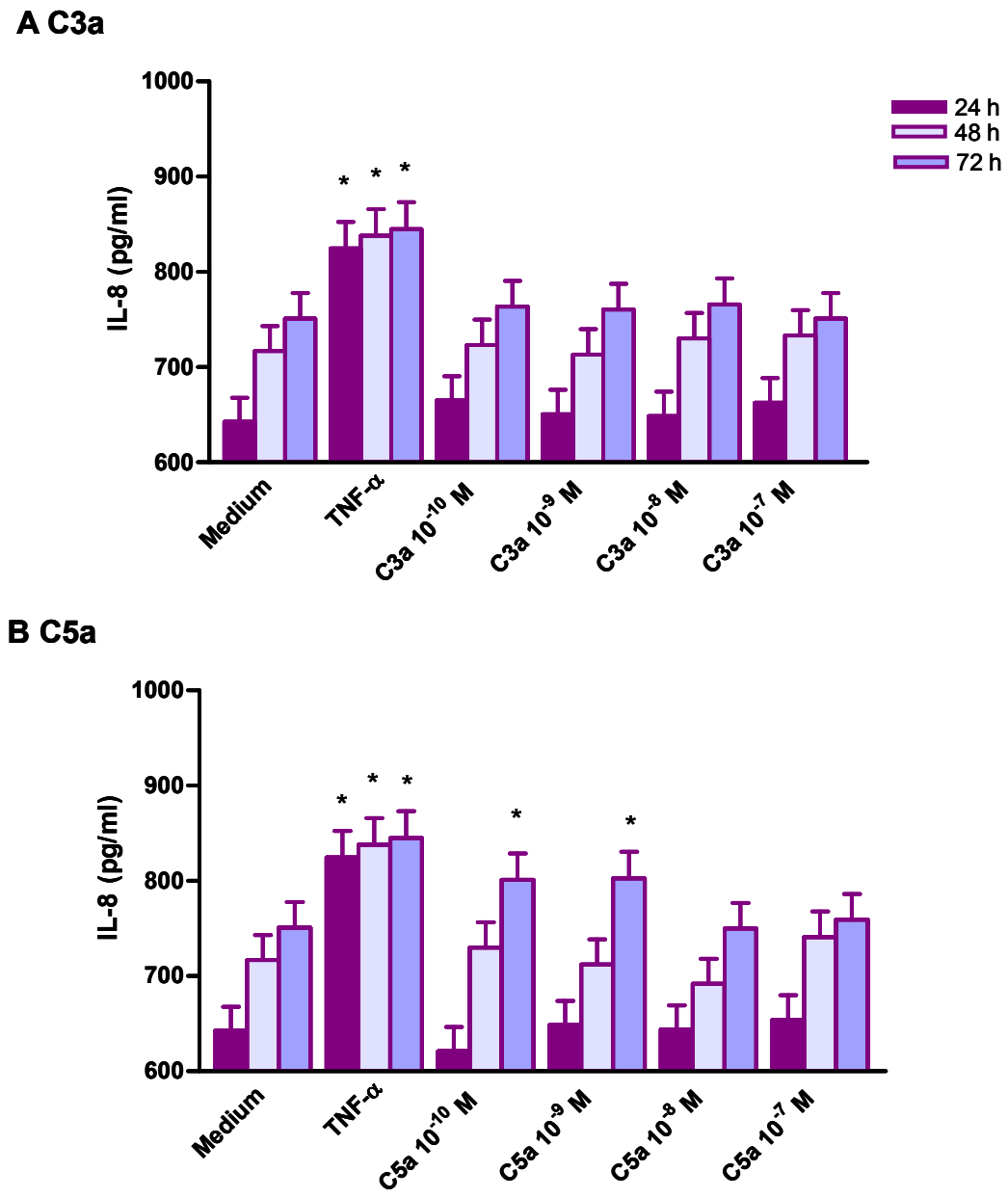

Figure 7.6 Effects of C3a and C5a on IL-8 production in HUVEC. Cells were cultured as described in Chapter 2: Materials & Methods, section 2.1.4.1 and stimulated for 24-72 h with various concentrations of A) C3a and B) C5a ranging from 10^{-10} M to 10^{-7} M, and TNF-α at 10 ng/ml as a positive control. Supernatants were harvested and IL-8 expression was measured by ELISA (refer to Chapter 2: Materials & Methods, section 2.5.3 for details of ELISA method). Data are expressed as the mean ± SEM of 3 independent experiments, * $p<0.05$ vs medium control at the same time point (Student's t-test).

7.2.5 FGF2 protein expression by endothelial cells

The effects of C3a, C5a, the Th2 cytokines IL-4 and IL-13 and the proinflammatory cytokine TNF-α on the expression of FGF2 by HPMEC was studied using ELISA (Figure 7.7). HPMEC spontaneously produced FGF2 in culture, and all of these mediators inhibited the production of FGF2 by HPMEC under the conditions employed.

7.2.6 Detection of FGFR1 in endothelial cells

The effects of C3a, C5a and the Th2 cytokines (IL-4 and IL-13 at 10 ng/ml) on the expression of FGFR1 in HUVEC was investigated by western blotting (see Chapter 2: section 2.5.4 for western blotting method). Neither the anaphylatoxins nor the cytokines were found to induce detectable expression of this growth factor receptor (data not shown).

7.2.7 VEGF protein expression by endothelial cells

The effects of C3a and C5a on the expression of VEGF in HUVEC were examined by ELISA. The anaphylatoxins were unable to induce detectable production of VEGF.

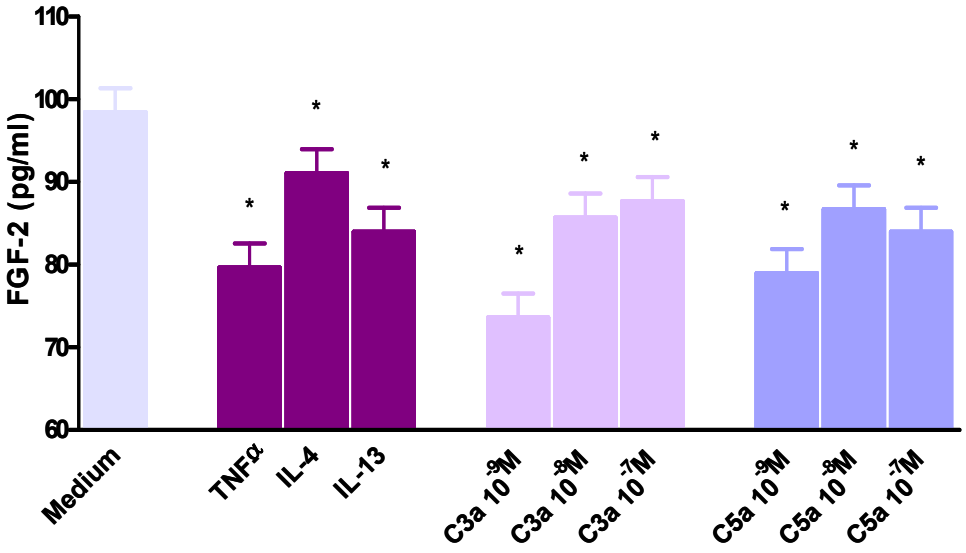

Figure 7.7 Effects of inflammatory cytokines, C3a and C5a on FGF2 expression by HPMEC. Cells were cultured and stimulated for 48 h with various concentrations of C3a and C5a ranging from 10^{-9} M to 10^{-7} M and Th2 cytokines and TNF-α at 10 ng/ml. Supernatants were harvested and FGF2 expression was assessed by ELISA as described in Chapter 2: Materials & Methods, section 2.5.3. Data are expressed as mean ± SEM of 3 independent experiments, * $p<0.05$ vs medium control (Student's t-test).

7.3 Discussion

In this chapter are presented investigations into possible effects of Th2 remodelling cytokines and anaphylatoxins on endothelial cells which may be relevant to remodelling processes in asthma.

It was confirmed that the complement receptors C3aR and C5aR are expressed on endothelial cells of pulmonary and umbilical vein origin. The expression of C3a and C5a receptors on HUVEC has previously been reported and this observation has been confirmed and extended here to include HPMEC (Monsinjon *et al.* 2003). The presence of these receptors indicates the potential for endothelial cells to respond to anaphylatoxins.

HUVECs are the most extensively studied type of endothelial cell, primarily because they are relatively easy to isolate and culture. Somatic microvascular endothelial cell types, which have recently become available, are not thoroughly characterised, although they are interesting from a physiological point of view (Boisen *et al.* 2010). Morphologically, HUVEC and HPMEC cultures are quite similar with slight variations in shape from the traditional cobblestone appearance to more of a spindle shape, although the degree of confluence in the cultures can influence the cell shape (Boisen *et al.* 2010). On the other hand HUVEC appear to senesce more rapidly than HPMEC in vitro, so the former have to be used early, typically after 2-3 passages in vitro following isolation, whereas HPMEC grow more slowly and require more passages in vitro to raise sufficient cell numbers.

In this study HPMEC were used for proliferation and growth factor production experiments whereas HUVEC were used to study IL-6 and IL-8 production

because Monsinjon and colleagues found anaphylatoxins to increase their production and we wanted to replicate this before using primary cells. There is some evidence that endothelial cell properties vary according to their site of origin (Burg *et al.* 2002) or even the size of the vessels from which they are derived (Meyrick, Christman, & Jesmok 1991). It would have been more satisfactory, therefore, to have used HPMEC throughout or at least compared these with HUVEC in each of the functional experiments.

The literature indicates that, of the fibroblast growth factors, FGF2 seems to be most highly expressed in the vascular compartments of the lung (Powell *et al.* 1998;Marek *et al.* 2009). FGF2 is an extremely important stimulator of angiogenesis, endothelial cell growth and the remodelling of ECM proteins through which new vessels grow (Desouza, Gerety, & Hamel 2009). Correspondingly, the remodelling cytokines IL-4 and IL-13 upregulated FGF2 mRNA expression early after stimulation of endothelial cells but this was accompanied by reduced release of FGF2 protein. Although mRNA encoding FGFR1, the FGF2 receptor was detectable in these cells it was not possible to detect expression of the immunoreactive protein. C3a and C5a did not significantly alter the production of mRNA encoding FGF2 and FGFR1, although there was a trend for an increase, but as with IL-4 and IL-13 production of FGF2 protein was actually reduced. The reason for this paradox is not clear.

It is intriguing that both C3a and C5a along with IL-4 and IL-13 appeared to reduce spontaneous FGF2 protein production by endothelial cells in vitro. This suggests the possibility that C3a and C5a may actually reduce angiogenesis and this deserves to be explored further both *in vitro* and *in vivo*.

VEGF is also an important remodelling factor influencing endothelial cell growth. VEGF and FGF are among the most important growth factors for endothelial cells (Desouza, Gerety, & Hamel 2009). VEGF is a powerful mitogen for endothelial cells and has been shown to accelerate endothelial regrowth and attenuate intimal hyperplasia post angioplasty (Ferrara 2005). In the present study the anaphylatoxins were unable to induce detectable VEGF protein production by HUVEC measured by ELISA.

C5a, at least at low nanomolar concentrations, but not C3a increased IL-8, but not IL-6 production by HUVEC. These data differ from those of Monsinjon and colleagues who reported that both C3a and C5a stimulation of these cells increased their expression of IL-8 (Monsinjon *et al.* 2003). Other stimuli have been described to increase IL-8 expression by endothelial cells. Notably IL-33, a newly identified member of the IL-1 family, exerts this effect through interaction with the endothelial cell ST2 receptor which is itself induced by Th2 cytokines (Aoki *et al.* 2010;Yagami *et al.* 2010).

Although outside the scope of the studies here, anaphylatoxins such as C5a may also influence endothelial cell function in inflammation by inducing leukocyte rolling/adhesion molecules such as P-selectin which are important in inflammatory cell recruitment (Bloemen *et al.* 2007). Again a variety of cytokines, including TNF-α, IFN-γ, IL-4 and IL-13 also increase the endothelial expression of adhesion molecules and may be responsible for the delayed leukocyte recruitment that characterises the “late phase” of acute allergic reactions (Pober *et al.* 1986;Bochner *et al.* 1995). Nevertheless, in spite of extensive data about the existence of endothelial cell/leukocyte adhesion molecule pairs, the extent to which their expression has an impact on the course of asthma awaits confirmation (Bloemen *et al.* 2007).

Other possible effects of anaphylatoxins on endothelial cells outside the scope of the present experiments include the possibility that they may regulate eNOS production and thus local vascular tone (Gifford *et al.* 2004) and matrix metalloproteinases (Mauro, Buscemi, & Gerbino 2010). In addition, preliminary experiments suggest that endothelial cells may release key asthma-relevant cytokines such as GM-CSF (Burg *et al.* 2002).

The data show that endothelial cells of diverse origin express receptors for the anaphylatoxins C3a and C5a and that these may influence cellular proliferation and regulate the production of a number cytokines and growth factors relevant to inflammation and tissue remodelling in asthma and other diseases. It is particularly intriguing that complement may under certain conditions be able to inhibit endothelial growth factor production and thereby, putatively, angiogenesis.

Chapter 8: Summary & Conclusion

8.1 Summary & Conclusion

At the outset of this work, in order to further investigate the possible role of complement and its receptors in asthma pathogenesis it was hypothesised that:

- There is elevated expression and or deposition of the complement fragments C3, C3d and C5b-9 in the bronchial mucosa of asthmatics compared to controls;
- There is elevated expression/deposition of the complement fragments C3, C3d and C5b-9 in the bronchial mucosa of atopic asthmatics following bronchial allergen challenge;
- C3a and C5a play a role in effecting airway remodelling by acting on structural cells, including epithelial cells, endothelial cells, fibroblasts and smooth muscle cells to induce remodelling changes or production of remodelling mediators.

To address these hypotheses, experiments described in chapter 3 assessed the deposition/expression of some complement fragments, both anaphylatoxin receptors in the bronchial mucosa of asthmatics compared to controls, and in addition in a group of mild, atopic asthmatics before and after allergen bronchial challenge. I also measured the numbers of FGF2 immunoreactive cells in the epithelium and submucosa of the asthmatics “at baseline” compared to controls. Experiments described in chapters 4-7 assessed effects of anaphylatoxins on key structural cells involved in airways remodelling in asthma.

The histological data somewhat supported the first hypothesis: the most striking finding was the expression of C3a and C5a receptors by at least a subpopulation of a wide variety of structural cell types within the bronchial mucosa, consistent with at least the propensity of these cells to respond to

complement fragments. This was confirmed by subsequent *in vitro* experiments, which clearly showed that lung epithelial, fibroblast, smooth muscle and endothelial cells express anaphylatoxin receptors. With regard to expression of complement fragments themselves, the picture was less clear and hampered by the lack of availability of a full range of suitable reagents. This necessitated study of the expression of C3, C3d and C5b-9 as surrogates of C3a and C5a production and while there is some justification for this, as expanded in chapter 3, it is not a direct measurement of local C3a and C5a expression, which may be particularly pertinent to dynamic situations such as following allergen challenge where changes in local expression of complement components could in theory vary quite substantially with time. Nevertheless the data do provide some circumstantial evidence for involvement of some of the components of the complement cascade in asthma in the sense that elevated C3 and C5b-9 immunoreactivity was observed in the epithelial, smooth muscle and submucosal regions of sections of bronchial biopsies from asthmatics compared to controls. Expression of C3d and C3aR immunoreactivity were likewise elevated although only in epithelium and smooth muscle respectively. On the other hand, correlation with disease severity was poor and might have been influenced by variable anti-asthma therapy, since the effects, if any, of anti-asthma medications on the production and expression of complement components within the bronchial mucosa are unknown. Furthermore, it is impossible to determine from histological studies whether production of complement fragments reflects local synthesis, in which case overall production might be expected to reflect the numbers of cells involved in the production, or binding of components delivered through the circulation. Finally, since the possible functional effects of complement components in the asthmatic bronchial mucosa are unknown it is difficult to envisage, at our present stage of

knowledge, physiologically relevant functional “readouts” of complement activity other than the rather dubious one of static disease severity.

With regard to correlation of disease severity with expression of complement components, the second hypothesis above was formulated to explore the effects of acute exacerbation of asthma by allergen challenge on local expression of complement components. As with the static experiments, changes in expression of the measurable complement components were not at all impressive following challenge; again it was possible to explore these changes at only one single time point (24 hours) following challenge and it could be argued that, in terms of possible early complement activation following challenge and the peak influx of inflammatory cells, this time point may have been too late.

With regard to the third and final hypothesis, the testing of which is reported in chapters 4-7, experiments with cultured cells were variable in substantiating the hypothesis. This is partly because, owing to the great difficulty in outgrowing primary structural cell lines from biopsies, in many cases the total numbers of experiments which could be performed within a reasonable time (and expense) frame was limited and interpretation therefore hampered by lack of statistical power. This is particularly true of the experiments addressing the effects of C3a and C5a on structural cellular proliferation, and exacerbated by the relatively low sensitivity and higher inherent variability of the colorimetric proliferation assay. Thus while C3a clearly increased fibroblast and endothelial cell proliferation at a range of physiological concentrations, its effects were less clear on smooth muscle and epithelial cell proliferation, probably owing to lack of statistical power. In contrast the effects of C5a on proliferation of all four of the structural cell types were equivocal, although by the same argument this could also reflect statistical under powering.

Apart from proliferation of airways structural cells, which is considered a key feature of the “remodelling” process, another focal point for investigation of the effects of C3a and C5a on remodelling emerged as their effects on the production of remodelling growth factors, and in particular FGF2 by the airways structural cells. As has been emphasised previously in the relevant chapters, FGF2 is one of the key remodelling growth factors implicated in mucosal remodelling and therefore arguably an important “readout” for the effects of complement complements on remodelling processes. Furthermore, all of the airways structural cell types studied in this thesis were capable of spontaneous FGF2 release in culture at the protein level. Fascinatingly, many, but not all of the observed effects of C3a and C5a on FGF2 production were inhibitory. Again, interpretation was somewhat impaired by the small numbers of experiments reducing statistical powering. Thus C3a clearly inhibited the release of FGF2 protein by bronchial epithelial cells; this was less clear in the case of C5a because of the limited numbers of possible experiments. While C3a appeared to increase FGF2 production by lung fibroblasts, C5a appeared to do the reverse although the poor quality of the concentration-response relationship in this particular experiment suggests a need for caution and the necessity for further verification. Both C3a and C5a inhibited FGF2 production by airways smooth muscle cells and endothelial cells, although again the poor concentration/response relationship observed with C5a in the case of endothelial cells suggests a need for caution pending further experiments.

Another area which will require future further clarification is the relationship between FGF2 mRNA and protein synthesis: while in many of the experiments the anaphylatoxins were observed to reduce FGF2 mRNA and protein expression in concert, this was not always the case: for example, in chapter 7

(endothelial cells) C3a appeared to increase production of FGF2 mRNA at the time points measured, whereas release of FGF2 protein in culture was reduced. These discrepancies will be fully clarified only when the full time course of alteration of FGF2 mRNA expression by complement components in the various target cells is fully understood (using sufficient numbers of experiments to ensure statistical power), as well as the dynamics of how changes in mRNA synthesis affect protein synthesis and whether all synthesised protein is secreted into culture supernatants. The pathophysiological consequences of these phenomena remain to be explored but clearly the data show that complement components may both promote and inhibit local production of key remodelling mediators, providing support for hypothesis three above.

In addition, the data suggest that C3a at a high physiological concentration can increase IL-6 production by fibroblasts while C5a can increase IL-8 production by endothelial cells. Thus the effects of anaphylatoxins may not be limited to regulation of growth factor expression, although again the pathophysiological consequences, if any, of these observations remain to be explored.

The main significance of this work is that it has shed light on the nature of expression of complement fragments, anaphylatoxin receptors and the growth factor FGF2 in the bronchial mucosa using an *in vivo* approach. The *in vitro* data have extended the functional implications of these observations to show that anaphylatoxins may influence a variety of key remodelling mechanisms in the asthmatic bronchial mucosa. The effects of the anaphylatoxins on the structural cells synthesising the various mediators that were explored in the chapters of this thesis are summarised in Figure 8.1.

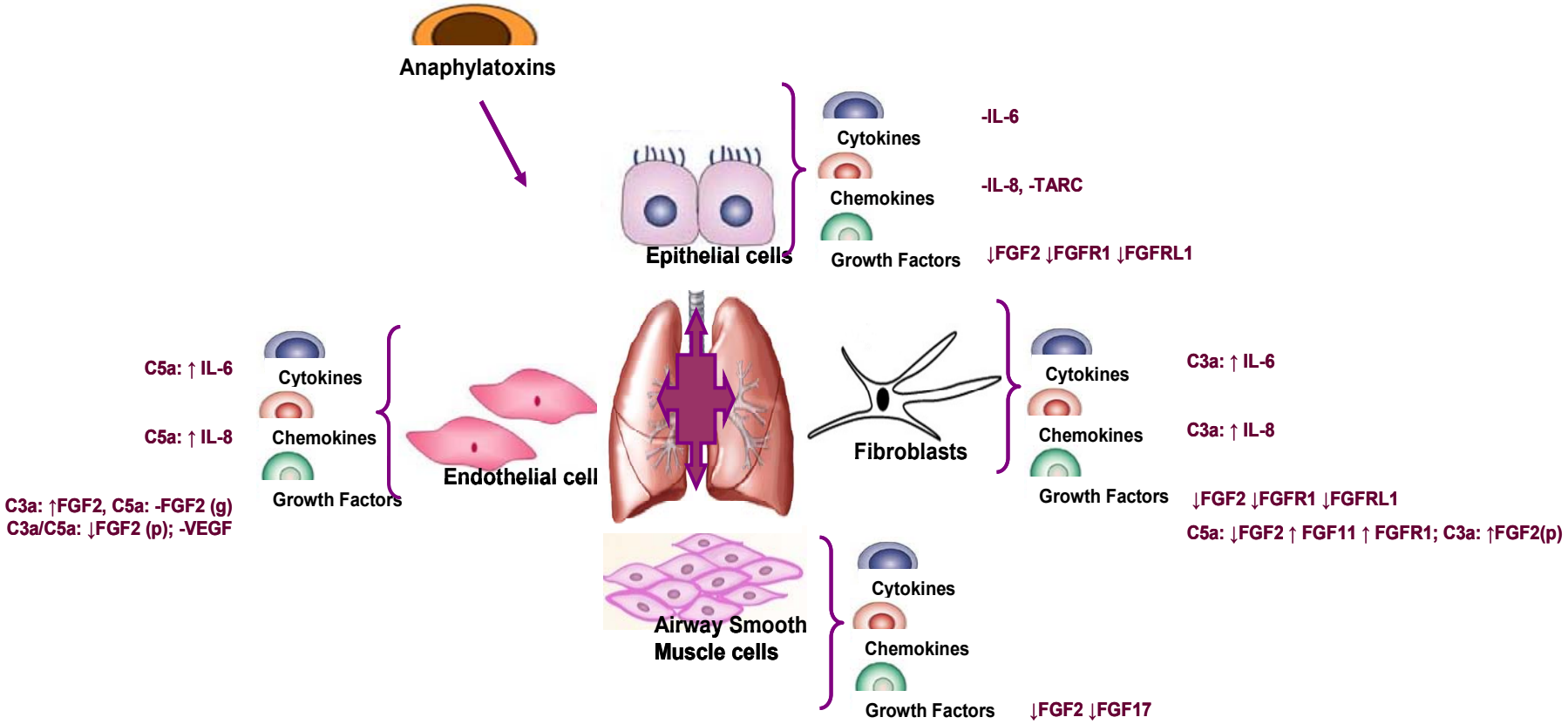

Figure 8.1 Potential effects of anaphylatoxins on structural cells of the airways as revealed by the work in this thesis.

8.2 Future Directions

The results of the present study motivate research in several different avenues. Given that they express appropriate receptors for, and are capable of physiological responses to the anaphylatoxins, the effects of anaphylatoxins on the four structural cell types looked at in this thesis surely merits further, in depth exploration under the umbrella of “remodelling”.

It will be appreciated from the work presented in this thesis that there are few established and readily available techniques of measuring complement components, and this deserves development (for example detection in induced sputum, bronchial brushings etc. as well as histologically in tissues).

One direction for the future would be to expand and amplify the experiments presented in this thesis to clarify some of the equivocal effects suggested by some of the data, and to allow a more global analysis of the effects of anaphylatoxins on purified populations of target structural cells using, for example, microarray analysis. This was attempted in a preliminary form when screening for effects of the anaphylatoxins on FGF production by airways structural cells in this thesis but could be expanded. Excessive mucus production by epithelial cells is another feature of remodelling and so it would be worthwhile exploring the effects of the anaphylatoxins on the expression of mucin genes. Studies on smooth muscle cells and fibroblasts could further be expanded by exploring possible effects of anaphylatoxins on myofibroblast differentiation (marked by the induction of smooth muscle actin) and their synthesis of interstitial proteins such as collagen and fibronectin. Since angiogenesis is also an important feature of airways remodelling; direct effects of the anaphylatoxins on angiogenesis *in vitro* could be sought, and any effects

clarified by investigation of effects on the production by endothelial cells of relevant mediators such as VEGF and its receptors.

In addition to looking at expression of complement components in the bronchial mucosa, it would also be of interest (and a challenge) to search for evidence that structural cells in asthma can actually manufacture any of the complement components C1-C9. This could be done in various ways, for example by comparing the gene expression profiles of asthmatics and normal control airways structural cells outgrown *ex vivo* as mentioned, although this would be a greater challenge than usual because the production of complement components such as anaphylatoxins can be triggered by a variety of pathways and production depends on the activities not of single enzymes but on cascades of enzymes.

To further build on the research presented here, it would be useful to extend the methods used for example to more sophisticated cell culture systems (for example recreating the air interface of bronchial epithelial cells or co-culturing epithelial cells and fibroblasts), as well as further exploiting the use of animal models, either with relevant gene deletions or by using complement antagonists (for example the data in this thesis suggest that it would be interesting to observe the effects of C3a gene deletion/pharmacological blockade on chronic remodelling changes in the airways, of which there are now several animal "models").

Above all it would be highly desirable to understand the possible role of complement components in asthma to the extent that biomarkers of their activity, either systemic or mucosal, can be used to correlate their activity with airways remodelling in asthma in the longer term.

The pathogenesis of asthma and associated airways remodelling remains a challenging subject with much more work still to be done. The work presented in this thesis has only begun to scratch the surface of investigation of the possible role of complement components; nevertheless it has made a significant contribution to new knowledge, principally by highlighting the possibility that complement components can be added to the list of many local influences which may drive remodelling changes, either directly or indirectly, in the asthmatic bronchial mucosa. It has been demonstrated that a surprisingly wide range of structural cells in the bronchial mucosa express anaphylatoxin receptors and posses at least the propensity to synthesise complement components. In a variety of airways structural cells, the anaphylatoxins exert measurable effects on the production of a range of remodelling and pro-inflammatory mediators, as well as cellular proliferation, suggesting a significant and long term role in airways changes in chronic asthma.

Chapter 9: Reference List

Reference List

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